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13. ABSTRACT *(Maximum 200 Words)*

These studies are designed to determine whether ethanol antagonizes the ability of the skeleton to adapt to increased mechanical usage. Ethanol reversibly alters the biophysical properties of cell membranes. The overall hypothesis to be tested in adult rats is that these membrane changes disrupt essential cell signaling pathways for one or more cytokines, growth factors and polypeptide hormones that regulate bone modeling and remodeling. This report summarizes our progress from 01 September 1999 to 31 August 2000. During Year 2 of the award we have continued analysis of experiments performed in Year 1 related to Tasks 1-4 and 8. Additional experiments were performed to accomplish Task 8. Progress was also made on Tasks 6, 7, and 8. The new studies are directed toward determination of the effects of ethanol on: the skeletal readaptation to normal weight bearing following unloading (Task 6), skeletal adaptation to treadmill running (Task 7), PTH-induced increases in mRNA levels for bone matrix proteins (Task 8), and PTH-induced increases in osteoblast number and bone formation (Task 9).

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(4) INTRODUCTION

Chronic alcohol abuse is an important risk factor for osteoporosis. The ultimate goal of this research is to identify the cellular and molecular mechanisms responsible for mediating ethanol's dose- and time-dependent actions on bone turnover, mass, architecture, and strength. It is well established that ethanol reversibly alters the biophysical properties of cell membranes and in doing so disturbs normal membrane function. The proposed studies in young adult rats will test our working hypothesis that these membrane changes disrupt essential cell signaling pathways for one or more bone cells "coupling" factors and/or polypeptide hormones that regulate bone modeling and remodeling. These changes are postulated to lead to the bone loss associated with chronic alcohol abuse. If our hypothesis is correct, then ethanol antagonizes the ability of the skeleton to respond to weight bearing because the signal transduction pathways for mechanical signals require peptide signaling molecules as intermediates. This latter effect of ethanol to reduce the ability of the skeleton to adapt to increased mechanical stress would be especially detrimental during rigorous military training.

(5) BODY

Introduction

Progress relevant to Tasks 1-5 as well as Task 8 were reported in the Year 1 progress report. Data analysis was continued in Year 2 and abstracts and manuscripts describing this work were submitted in Year 2 (see item 8, reportable outcomes and Appendices). Additional work directed toward accomplishing Tasks 6-9 were performed.

We will discuss the progress for each Task separately. Appendix 1 lists experiment number, title, and Task(s).

Task 2

The goal of this Task was to determine the time course effects of alcohol on gene expression of bone matrix proteins and signaling molecules. The original goals of this Task were accomplished by the end of year 1. However, continuing to investigate mRNA isolated during the course of performing these studies using cDNA microarrays. This technology was not available when the proposal was submitted and results of this analysis are likely to provide additional insight into the mechanism for the detrimental actions of alcohol on bone metabolism. Using this method we have shown that alcohol result sin tissue specific effects on gene expression. Furthermore, alcohol influences the expression of many more genes and signaling pathways in bone than formally suspected. The details of the experimental design, results and interpretation can be found in Appendices 2 and 3.

Task 6

The goal of this Task was to determine the effects of ethanol on skeletal adaptation to normal weight bearing following unloading. Members of Dr. Turner's laboratory performed a hindlimb unloading study in Dr. Emily Morey-Holton's laboratory at NASA-Moffett Field in order to

transfer expertise in using this model system to Mayo. Mayo engineering has constructed a prototype hindlimb unloading cage. The design has received approval and 30 cages will be manufactured this fall. We anticipate performing the hindlimb unloading studies in Year 3, as originally proposed.

Task 7

The goal of this Task was to determine the effects of ethanol on the skeletal adaptation to treadmill running. The treadmill running equipment has been procured and tested. Rats were ordered and adaptation to alcohol and treadmill running performed. The animal experiment has been started and will be completed in December 2000.

Task 8

The goal of this Task was to determine the effects of consumption of ethanol on PTH-induced increases in mRNA levels for bone matrix proteins. Two new studies have been completed (see Appendix 1).

One study investigated the effects of concurrent daily administration of alcohol and PTH. Alcohol was administered orally to simulate moderate drinking (1 g/alcohol/kg body weight). Moderate alcohol did not antagonize the stimulatory effects of PTH on steady-state mRNA levels for bone matrix proteins. The details of the experimental design, results and interpretation of this study can be found in Appendix 2.

The second study investigated the effects of prior consumption of ethanol on PTH-induced increases in bone matrix proteins. Alcohol was administered to rats in their diet (36% caloric intake) for one week prior to administration of alcohol (80 µg/kg/d for one week). Alcohol feeding was continued during the PTH treatment interval. Alcohol treatment significantly reduced the anabolic effects of PTH on osteoblast number and bone formation (see Appendix 4 for details).

Task 9

The goal of this task was to determine the effects of PTH-induced increases in osteoblast number and bone formation. Moderate alcohol consumption had not effect on PTH-induced increases in bone formation and osteoblast number. In contrast, chronic alcohol abuse reduced bone formation and osteoblast number and antagonized the PTH induced increases in bone formation and osteoblast number. The details of the experimental design, results and interpretation of these studies can be found in Appendices 2 and 4.

An additional *in vitro* study was performed to determine whether the inhibitory effects of alcohol on bone cell number and activity is due to a direct toxic action on the osteoblast. These results demonstrate that relevant concentrations of alcohol have little or no direct effects on osteoblast proliferation or expression of differentiated function. The details of these studies can be found in Appendix 5.

(6) KEY RESEARCH ACCOMPLISHMENTS

The detrimental effects of chronic alcohol abuse on the skeleton of humans and laboratory animals are well established (1-9). The present studies demonstrate that concurrent moderate alcohol consumption has no effect on PTH-induced bone formation, whereas chronic alcohol abuse significantly impairs the beneficial skeletal response to PTH. Additionally, the studies performed during year 2 strongly suggest that the detrimental effects of alcohol on bone cells are reversible. The PTH response, although impaired by alcohol abuse, was sufficiently robust to effectively counteract the detrimental effects of alcohol. Furthermore, *in vitro* studies failed to demonstrate a direct toxic effect of alcohol on osteoblasts.

(7) REPORTABLE OUTCOMES

Maran A, Zhang M, Spelsberg TC, Turner RT: The dose response effects of ethanol on human fetal osteoblastic cell line. J Bone Miner Res, In press.

Turner RT, Kidder LS, Kennedy A, Evans GL, Sibonga JD: Moderate alcohol consumption suppresses bone turnover in adult female rats. J Bone Miner Res, In revision.

Turner RT: Skeletal response to alcohol. Alcoholism: Clinical and Experimental Research, In press.

Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD: Animal models for osteoporosis. Reviews in Endocrine and Metabolic Disorders, In press.

Zhang M, Kidder LS, Patterson-Buckendahl P, Kennedy AM, Evans GL, Maran A, Sibonga JD, Turner RT: Moderate alcohol suppresses bone turnover in adult female rats. 22nd Annual Meeting of the American Society for Bone and Mineral Research, September 2000, poster presentation.

(8) CONCLUSIONS

Our year 2 results are consistent with the initial hypothesis. We do not anticipate any need for major changes in either the working hypothesis or specific aims of this proposal. We have clearly shown that alcohol abuse antagonizes the skeletal response to PTH. Nevertheless, PTH was effective in increasing bone formation in alcoholic rats to values which exceeded normal. Thus, an important unanticipated finding of our research is that PTH treatment may be effective in treatment of alcohol-induced osteoporosis.

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(10) APPENDICES

Appendix 1: Summary of Experiments Performed

Appendix 2: Turner RT, Evans GL, Zhang M, Maran A, Sibonga JD: Co-administration of alcohol does not prevent parathyroid hormone-induced bone formation. Draft manuscript

Appendix 3: Maran A, Zhang M, Turner RT: Organ-specific effects of alcohol on gene expression. Draft manuscript

Appendix 4: Turner RT, Evans GL, Zhang M, Sibonga JD: Effects of parathyroid hormone on bone formation in alcoholic rats. Draft manuscript.

Appendix 5: Maran A, Zhang M, Spelsberg TC, Turner RT: The dose response effects of ethanol on human fetal osteoblastic cell line. J Bone Miner Res, In press.

Appendix 6: Turner RT, Kidder LS, Kennedy A, Evans GL, Sibonga JD: Moderate alcohol consumption suppresses bone turnover in adult female rats. J Bone Miner Res, In revision.

Appendix 7: Turner RT: Skeletal response to alcohol. Alcoholism: Clinical and Experimental Research, In press.

Appendix 8: Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD: Animal models for osteoporosis. Reviews in Endocrine and Metabolic Disorders, In press.

Appendix 9: Zhang M, Kidder LS, Patterson-Buckendahl P, Kennedy AM, Evans GL, Maran A, Sibonga JD, Turner RT: Moderate alcohol suppresses bone turnover in adult female rats. 22nd Annual Meeting of the American Society for Bone and Mineral Research, September 2000, poster presentation.

Appendix 1: Summary of Experiments Performed

Experiment #	Title	Task Number(s)
11*	Effects of Alcohol on Skeletal Adaptation to Treadmill Running	7
12	Effects of Concurrent Daily Administration of Moderate Alcohol and PTH on Bone Metabolism	8,9
13	Effects of Prior Consumption of Ethanol on PTH-induced Increase in Bone Matrix Proteins	8,9
14	Effects of Alcohol on Growth and Differentiation of Human Osteoblasts	9
* Experiment #s 1-10 were reported previously		

CO-ADMINISTRATION OF ALCOHOL DOES NOT PREVENT PARATHYROID HORMONE-INDUCED BONE FORMATION

Running Title: Alcohol and PTH

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ABSTRACT

Background: Alcohol suppresses bone formation in humans and laboratory animals and as a consequence induces osteopenia. It is not clear whether these detrimental effects are reversible. The present investigation in rats was designed to investigate the effects of alcohol on the skeletal response to parathyroid hormone (PTH), a potent anabolic hormone in bone being investigated as a therapeutic agent to reverse bone loss .

Methods and Results: Alcohol was administered daily to 6-month-old female rats by gavage (1 g/kg/d) or in a liquid diet (35% caloric intake) to simulate moderate and abusive drinking, respectively. Human (1-34) PTH (80 μg/kg/d) was administered sc for one week. Even moderate alcohol consumption resulted in dramatic changes in gene expression in proximal tibial metaphysis as determined by microarray and Northern analyses, including decreases in steady-state mRNA levels for type 1 collagen, osteonectin and osteocalcin. PTH increased histomorphometric indices of bone formation (mineralizing perimeter, mineral apposition rate and bone formation rate), as well as steady-state mRNA levels for the three bone matrix proteins. This potent anabolic response was not influenced by co-treatment with the moderate dose of alcohol and was not prevented at the high dose.

Conclusions: We conclude that co-ingestion with moderate alcohol does not impair the stimulatory effects of PTH on bone formation. This is evidence that the effects of alcohol on the skeleton are reversible. Additionally, the positive effects on bone formation in rats consuming high concentrations of alcohol suggest that PTH may be useful as an intervention to treat alcohol-induced osteoporosis.

Key Words: bone histomorphometry, mineralization, rat tibia, bone resorption

INTRODUCTION

Alcohol abuse is an important risk factor for osteoporosis (Spencer et al., 1986; Bikle et al., 1985; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Schnitzler and Solomon, 1984; Diez et al., 1994; Bikle et al., 1993), the etiology of which is not completely understood. However, there is overwhelming evidence that bone formation is depressed in alcoholics (Bikle et al., 1985; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Nielsen et al., 1990; Labib et al., 1989; Rico et al., 1987). Additionally, administration of ethanol to healthy volunteers decreases serum osteocalcin, a biochemical marker of bone formation (Laitinen et al., 1994; Laitinen et al., 1991).

It is important to understand the mechanism for the inhibitory effects of ethanol on bone formation. If ethanol has a direct toxic effect on the osteoblast, as has been suggested (Rico et al., 1987), then drinking would be expected to prevent the skeletal response to agents which stimulate bone matrix synthesis. End organ resistance to endogenous anabolic hormones could also contribute to the etiology of alcohol-induced osteoporosis. Furthermore, anabolic agents used to treat osteoporosis might be less effective if administered in the presence of ethanol. To test the possibility that alcohol prevents the skeletal response to bone anabolic agents, we determined the effects of ethanol on the efficacy of parathyroid hormone (PTH)-induced bone formation in adult six-month-old female rats. Two models were studied: modest amounts of alcohol were administered periodically to model moderate drinking and large amounts of alcohol was added to the diet to simulate chronic alcohol abuse.

MATERIALS AND METHODS

Animals

The animal studies were approved by the Institutional Animal Care Committee.

Moderate Alcohol Model

Six-month-old female Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing an average of 286 ± 4 g (mean ± SE) were randomized and weight-matched into three groups (n=6 rats/group). Two of the groups were administered 1.0 ml of water by gavage immediately followed by sc injection of human (1-34) PTH (Bachem, Torrance, CA) at 80 µg/kg/d, or of vehicle (1 mM HCl, 2% heat-inactivated serum in sterile saline). This dose of PTH results in a near optimal stimulation of bone formation (Dobnig and Turner, 1997). The third group (PTH/ethanol) was administered 0.4 ml 95% ethanol diluted to 1.0 ml in water by gavage immediately followed by sc PTH injection (80 µg/kg/d). Chronic treatment with this dose of ethanol was previously shown to inhibit bone formation (Turner, et al., 1998). Rats were injected at the base of the tail with tetracycline-hydrochloride (Sigma, St. Louis, MO) at 20 mg/kg on the first day of treatment and with calcein (Sigma) at 20 mg/kg on the sixth day of treatment.

After 7 days of treatment, rats were anesthetized with a 10:1 mixture of ketamine (100 mg/ml) and xylazine (100 mg/ml) before sacrifice by decapitation. Blood was collected by trunk bleed and allowed to clot for serum separation. Sera were aliquoted and stored frozen at –70°C until assayed. Tibiae were defleshed and either frozen in liquid nitrogen for RNA analysis or fixed by immersion in 70% ethanol for bone histomorphometry.

Previous findings (Turner, et al., 1998) that administration of ethanol (1 g/kg/d) has important effects on bone metabolism were confirmed and extended by two additional experiments. In the first experiment, 12 six-month-old female rats were studied. Ethanol was administered to eight rats as described and the remaining rats received water only. The rats were sacrificed 24 h later and tibia excised as described for RNA analyses. The second experiment

was similar to the first, except five animals were included in each group and the animals were treated daily with alcohol or water for 7 d and sacrificed 24 h following the final dosing.

Alcohol Abuse Model

The question as to whether PTH stimulates bone formation in rats treated with higher doses of alcohol was addressed in a separate experiment. Three groups (N=10/group) of six-month-old female rats were studied; group 1 was fed the control liquid diet, group 2 the alcohol diet (35% caloric intake) and group 3 the alcohol diet plus PTH (80 μg/kg/d). The rats were adapted to the liquid diet with increasing concentrations of alcohol (groups 2 and 3) for one week as described (Kidder and Turner, 1998) prior to PTH treatment. The rats were given fluorochromes as described for the first experiment. After the seven day PTH treatment interval, the rats were sacrificed and tibia excised for histomorphometry as described for the first experiment.

Bone Histomorphometry

Tibiae were dehydrated by immersion in a series of increasing concentrations of ethanol, and embedded without demineralization in a methylmethacrylate mixture (methylmethacrylate:2-hydroxyethyl-methacrylate 12.5:1). Longitudinal sections were taken from the middle of the proximal tibia using a Reichert-Jung microtome (5 μm thick) and permanently mounted on slides for histomorphometric measurements. Cancellous bone measurements were performed in a standard sampling site located on the long axis of the bone, one millimeter from the epiphyseal growth plate at its most distal point. This site is distal to the primary spongiosa, within the secondary spongiosa, and extends bilaterally to exclude the endocortical edges. The sampling site encompassed a tissue area of 2.88 mm^2.

All histomorphometric measurements were carried out with a semi-automatic image-analysis system, which consisted of a Compaq computer interfaced with a microscope and image analysis software (OsteoMetrics, Inc., Atlanta, GA). Skeletal indices were measured by registering the movement of a digitizing mouse across a graphics tablet as a tracing was superimposed on an image of the section displayed on a video screen. The computer software recorded lengths and calculated the enclosed areas. The following histomorphometric indices were determined as described (Kidder and Turner, 1998): cancellous bone area, trabecular number, trabecular thickness, trabecular separation, mineralizing perimeter, mineral apposition rate, and bone formation rate (perimeter referent). Measurements were performed in accordance with standardized nomenclature and methods (Parfitt et al., 1987).

Northern Hybridizations

Total RNA was isolated from tibial metaphyses for Northern analysis of steady-state mRNA levels for selected bone matrix proteins. Metaphyses from frozen tibiae were individually crushed into powder using a Spex freezer mill (Edison, NJ) and total cellular RNA was extracted and isolated with a modified organic solvent method as reported (Cavolina et al., 1997). Ten μg of total RNA from each sample was denatured in 1M glyoxal, 50% dimethylsulfoxide 0.01 M NaH_2PO_4 at 52°C, separated by electrophoresis on a 1% agarose gel, transferred by capillary action in 20X SSC to nylon membranes (Amersham Hybond nylon membrane, Arlington Heights, IL) and crosslinked using a UV source (Stratagen, LaJolla, CA) before hybridization procedures. Membranes were prehybridized for 1-2 hrs at 65°C in a Rapid-Hyb Buffer (Amersham). Hybridization was carried out for 80 minutes in a buffer containing the above ingredients in addition to a minimum of $4x10^6$ cpm/ml ^{32}P-labeled cDNA for the following bone matrix proteins: prepro-α (I) subunit of type I collagen (collagen), osteocalcin and

osteonectin. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and by hybridization with ^{32}P-labeled cDNA for 18S ribosomal RNA. Representative Northern blots of bone extracts from ethanol and from PTH-treated rats are published (Dobnig and Turner, 1995; Turner, et al., 1998).

cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit (Amersham, Arlington Heights, IL). The membranes were washed for 30 minutes at RT in 3X SSC and for 15 minutes in 1X SSC at 43-65°C.

The mRNA bands on the Northern blots were quantitated by densitometric scanning (Molecular Dynamics Phosphor Imager, Sunnyvale, CA) and values normalized to 18S ribosomal RNA.

Gene Filter MicroArray

Gene filter microarrays were performed using rat gene filter 1 and software from Research Genetics (Huntsville, AL). To reduce the effects of individual differences in gene expression, total RNA (1 μg) isolated from bone pooled from five animals was used for cDNA synthesis. The synthesis of cDNA, prehybridization, hybridization and washings were performed as described in the manufacturer's protocol (Research Genetics). The radioactive filters were exposed for 48 hrs on a phosphor screen to get the phosphor image of the gel using the Cyclone Storage Phosphor system (Packard Instrument Company, Meriden, CT). The phosphor image was analyzed using Pathways software (Research Genetics) to determine gene specific changes.

Serum Chemistry

Total serum calcium, serum phosphorous, magnesium and creatinine were quantitated with a Hitachi 717 automated system (Hitachi, Hialeah, FL) by the Central Clinical Laboratory (Mayo Clinic, Rochester, MN).

Statistical Analysis

Values are expressed as mean ± standard error. Group comparisons were analyzed by Fisher's Protected Least Significant Difference post-hoc test following determination of significance by 1-way ANOVA. Significance was established at p values ≤ 0.05.

RESULTS

Neither alcohol nor PTH (moderate and alcohol abuse models) had an effect on body weight (data not shown). Similarly, static indices of cancellous bone architecture, including bone area, trabecular number, trabecular thickness and trabecular separation did not differ significantly among groups (data not shown).

As shown in Table 1 for the moderate alcohol model, PTH and PTH/ethanol had no effect on serum phosphorous, magnesium and creatinine. PTH had no effect on serum calcium, but PTH/ethanol resulted in a small increase compared to the vehicle-treated group.

As shown in Figure 1 for the moderate alcohol model, both treatments resulted in significant changes in dynamic bone histomorphometry in cancellous bone. PTH treatment resulted in an increase in bone formation rate (Figure 1A), an increase in mineralizing perimeter (Figure 1B), and no change in mineral apposition rate (Figure 1C). The effects of combination treatment (PTH/ethanol) on dynamic bone histomorphometry did not differ from PTH alone.

As shown in Figure 2 for the moderate alcohol model, both treatments resulted in significant changes in steady-state mRNA levels for bone matrix proteins. PTH treatment resulted in increases in mRNA levels for type 1 collagen (Figure 2A), osteonectin (Figure 2B), and osteocalcin (Figure 2C). The effects of combination treatment (PTH/ethanol) on mRNA levels for the bone matrix proteins did not differ from PTH alone.

A single oral administration of 1 g/kg of ethanol significantly reduced steady-state mRNA levels for type 1 collagen (Figure 3A), osteonectin (Figure 3B), and osteocalcin (Figure 3C).

After seven days of treatment, moderate ethanol resulted in changes in steady-state mRNA levels greater than 2.5-fold for 1047 genes out of 5531 unique cDNAs spotted on the microarray filter (Figure 4). The changes included known genes as well as expressed sequence tags. The changes included both increases and decreases. The known responding genes encode several classes of peptides, including structural proteins, enzymes, growth factors and receptors. A more complete analysis of these data will be published elsewhere.

The rats fed alcohol at 35% of their caloric intake to model chronic alcohol abuse consumed approximately 15 g/kg/d of ethanol. Bone formation was reduced in these animals to $20 \pm 10\%$ ($p < 0.001$) of the mean value for rats fed the control diet. PTH significantly increased bone formation in the rats fed alcohol compared to the alcohol group without PTH ($+ 1200 \pm 40\%$; $p < 0.001$). PTH also significantly increased bone formation compared to the control diet group ($+ 240 \pm 40\%$; $p < 0.001$). The detailed results of this study will be published elsewhere.

DISCUSSION

The rat appears to be a good laboratory animal for modeling alcohol-induced changes in human bone metabolism as well as the anabolic effects of PTH on bone. Chronic consumption of large amounts of alcohol results in disturbed mineral homeostasis in male and female rats similar to alcoholics (Peng and Gitelman 1974; Turner et al., 1987; Turner et al., 1991; Peng et al., 1988; Sampson et al., 1996; Hogan et al., 1992; Turner et al., 1988; Sampson and Spears, 1999; Sampson, 1999). As in alcoholics, alcohol depresses bone formation in rats (Turner et al., 1987; Turner et al., 1991; Sampson et al., 1996; Hogan et al., 1992; Sampson, 1999; Dyer et al., 1998). Pulsatile treatment with PTH increases bone formation in rats and humans (Dobnig and Turner, 1997; Mosekilde and Reeve, 1996). Thus, using rats to study the effects of PTH and alcohol on skeletal tissues is likely to produce results relevant to humans.

PTH treatment resulted in histomorphometric changes consistent with those reported in published studies, including increases in bone formation rate (Dobnig and Turner, 1995; Dobnig and Turner, 1997; Turner et al., 1998). The lack of an effect of PTH on bone area and architecture was anticipated because of the short duration of the study (Dobnig and Turner, 1995; Dobnig and Turner, 1997; Turner et al., 1998).

PTH increases bone formation in rats by increasing osteoblast number and to a lesser extent activity (Dobnig and Turner, 1997; Dobnig and Turner, 1995; Turner et al., 1998; Wronski et al., 1993; Liu and Kalu, 1990). In the current study, mineralizing perimeter was measured as an index of osteoblast number, and mineral apposition rate was calculated as an index of osteoblast activity. As previously shown, the increase in mineralizing perimeter was largely responsible for the PTH-induced increase in bone formation.

PTH treatment rapidly increases osteoblast number by targeting and activating a population of quiescent cells to express the osteoblast phenotype. We have termed this process

"modulation." The targets of this hormone's action are probably bone lining cells (Dobnig and Turner, 1995; Turner et al., 1998). Bone lining cells are derived from osteoblasts and are believed to play an important role in regulation of bone remodeling and calcium homeostasis (Mundy et al., 1990; Parfitt, 1989). PTH probably accomplishes this action by stimulating the production of cytokines and growth factors (e.g., IGF-I) positively associated with osteoblast differentiation and activity (Finskelstein, 1996).

The main study was performed in normal rats administered moderate alcohol. This model was designed to evaluate the possible antagonistic actions of ethanol on PTH-induced bone formation in a non-alcoholic population. As an important physiological regulator of bone turnover, PTH is being developed as a therapy to reverse postmenopausal osteoporosis. It is important, therefore, to establish whether moderate alcohol consumption antagonizes the skeletal response to PTH.

It is also relevant to assess the effects of PTH treatment on a model of excessive alcohol consumption because higher doses of ethanol known to decrease bone formation (Sampson, 1999; Sampson et al., 1996; Sampson and Spears, 1999; Turner et al., 1986; Turner et al., 1987; Turner et al., 1991) might antagonize PTH action. However, the present study performed in the rat model for chronic alcohol abuse clearly demonstrated that PTH can stimulate bone formation in rats exposed to high levels of alcohol.

Ethanol suppresses bone formation in rats and humans, by decreasing indices of osteoblast number and activity (Gonzalez-Calvin, et al., 1993; Lalor et al., 1986; Nielsen et al., 1990; Labib et al., 1989; Rico et al., 1987; Laitinen et al., 1994; Laitinen et al., 1991; Peng and Gitelman, 1974; Turner et al., 1987; Turner et al., 1991; Sampson et al., 1996; Hogan et al., 1992). However, there is very little direct evidence that either moderate or abusive alcohol

consumption has a toxic effect on cells of the osteoblastic lineage. Indeed, some studies suggest that moderate alcohol consumption increases bone mass (Laitinen et al., 1993; Feskanich et al., 1999; Orwoll et al., 1996; Laitinen et al., 1991). On one hand, prolonged exposure to high concentrations of ethanol reduced proliferation of osteosarcoma cells but did not induce cell death (Klein et al., 1996). Similarly, dose response studies using conditionally immortalized human osteoblastic cells (fHOB) demonstrated minimal direct effects of ethanol on cell survival (Maran et al., 1999). On the other hand, ethanol was shown to reduce in vivo (Turner et al., 1998) and in vitro (Maran et al., 1999) expression of growth factors and cytokines necessary to maintain the osteoblast phenotype, including IGF-I and TGF-ß.

Alcohol-induced reduction in the expression of cytokines and growth factors which maintain osteoblastic activity could result in modulation of the osteoblast phenotype to the quiescent (in regards to bone matrix production) bone lining cell phenotype. The postulated transformation would result in reductions in the apparent number of osteoblasts and osteoblast activity and an overall decrease in bone formation, changes consistent with the known effects of acute as well as chronic alcohol consumption (Turner et al., 1998). Thus, PTH might exert its beneficial effect on alcohol-treated rats by compensating for the inhibitory effects of alcohol on expression of cytokines and growth factors that maintain osteoblast activity. Further research is necessary to test this hypothesis.

The results of the present study support neither a direct toxic effect of ethanol on osteoblasts nor skeletal resistance to PTH because the anabolic effects of hPTH were not prevented by alcohol. The observed results support, but do not prove, the alternative explanation that ethanol modulates osteoblasts to express the bone lining cell phenotype. Whatever the

precise mechanism, we have shown that PTH can reverse the inhibitory effects of alcohol on bone formation.

In summary, the present study demonstrates that the anabolic effects of PTH on bone formation are not antagonized by concurrent exposure to moderate levels of alcohol. Also, much greater amounts of alcohol did not prevent the stimulatory effects of PTH on bone formation. These results showing that PTH increases bone formation in rats in which bone formation was suppressed by alcohol treatment provide evidence that ethanol inhibits bone formation by a reversible pathway. Long-term studies in osteopenic rats should be performed to evaluate the efficacy of PTH treatment as a therapy to reverse the detrimental skeletal effects of alcohol abuse.

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FIGURE LEGENDS

Figure 1: Effects of PTH and combination treatment with alcohol and PTH on cancellous bone histomorphometry. Values are mean ± SE; n = 6.

Figure 2: Effects of daily treatment for one week with PTH and combination treatment with alcohol by gavage and PTH on steady state mRNA levels for bone matrix protein. Values are mean ± SE; n = 4.

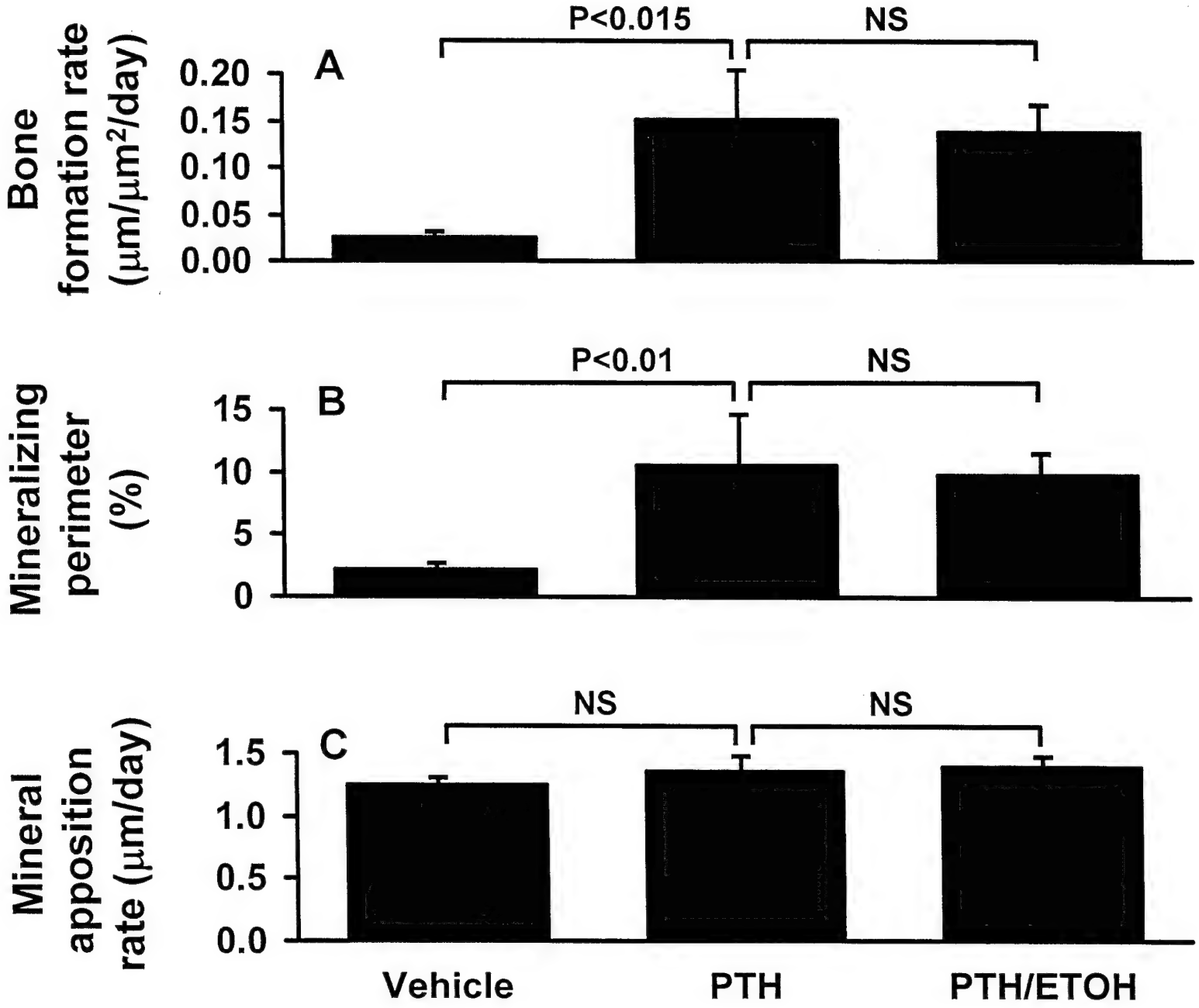
Figure 3: Effects of a single treatment with ethanol (1 g/kg) by gavage on steady-state mRNA levels for bone matrix proteins 24 hrs later. Values are mean ± SE; n = 8 for alcohol and 4 for control.

Figure 4: Phosphoimage of gene filter microarray. Gene expression in rats administered alcohol (1 g/kg) daily by gavage for one week was compared to rats receiving water only. The RNA was pooled from 4 rats in each treatment group. Each intersection of horizontal and vertical lines represents the position of a unique cDNA. Red dots identify genes for which mRNA was increased by alcohol whereas blue dots indicate genes that were decreased. Only genes which changed by $\geq$ 2.5 fold are shown.

Table 1. Influence of ethanol on PTH effects on serum chemistry.

TREATMENT	n	CALCIUM (mg/dl)	PHOSPHORUS (mg/dl)	MAGNESIUM (mg/dl)	CREATININE (mg/dl)
Vehicle	4	9.23±0.08	8.05±0.36	2.53±0.09	0.55±0.03
PTH	5	9.18±0.07	8.38±0.15	2.66±0.07	0.60±0.00
PTH/Ethanol	4	9.80±0.10*	7.75±0.17	2.53±0.05	0.60±0.00

Values are Mean ± SE. * $p < 0.05$ vs. vehicle



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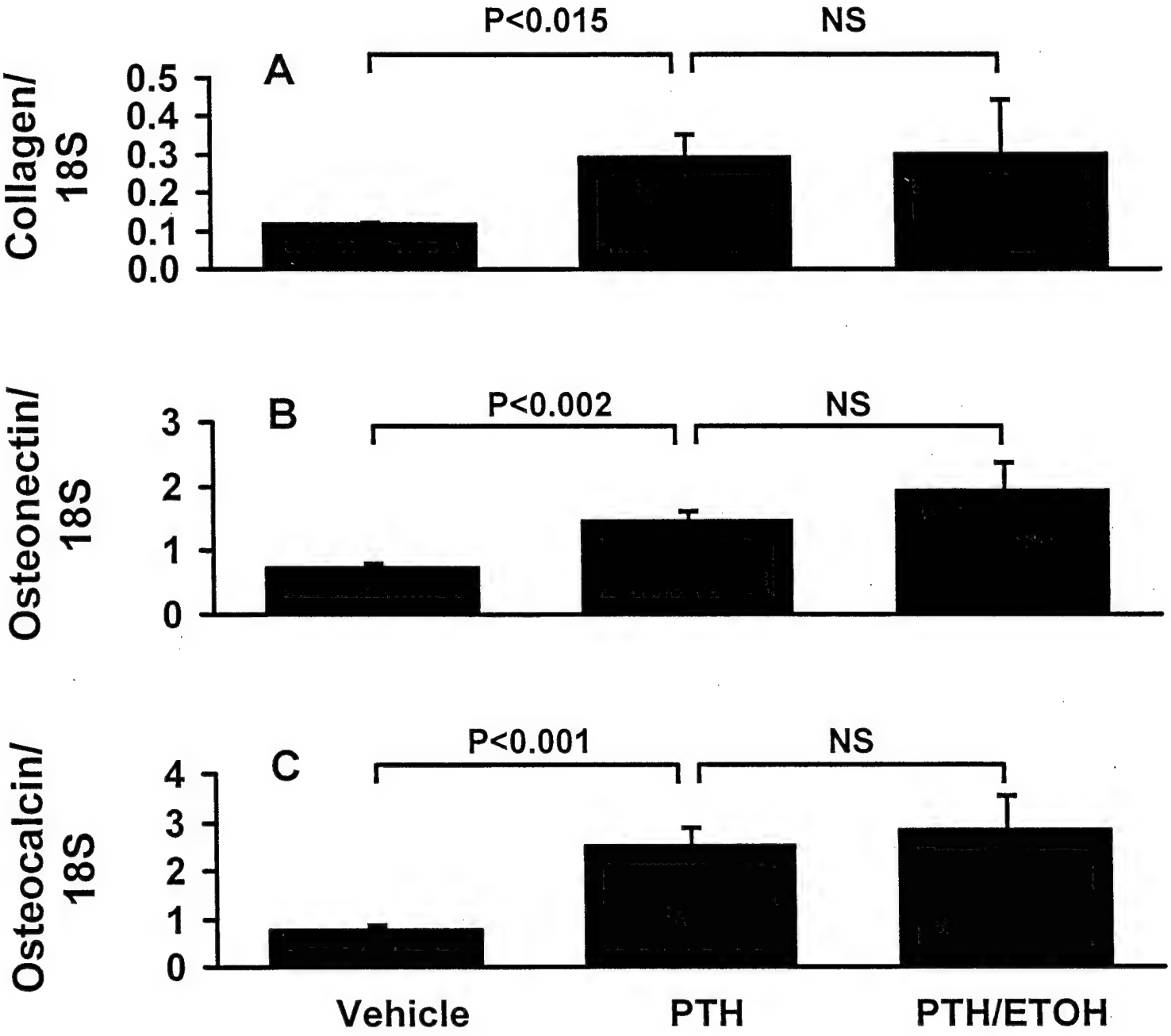

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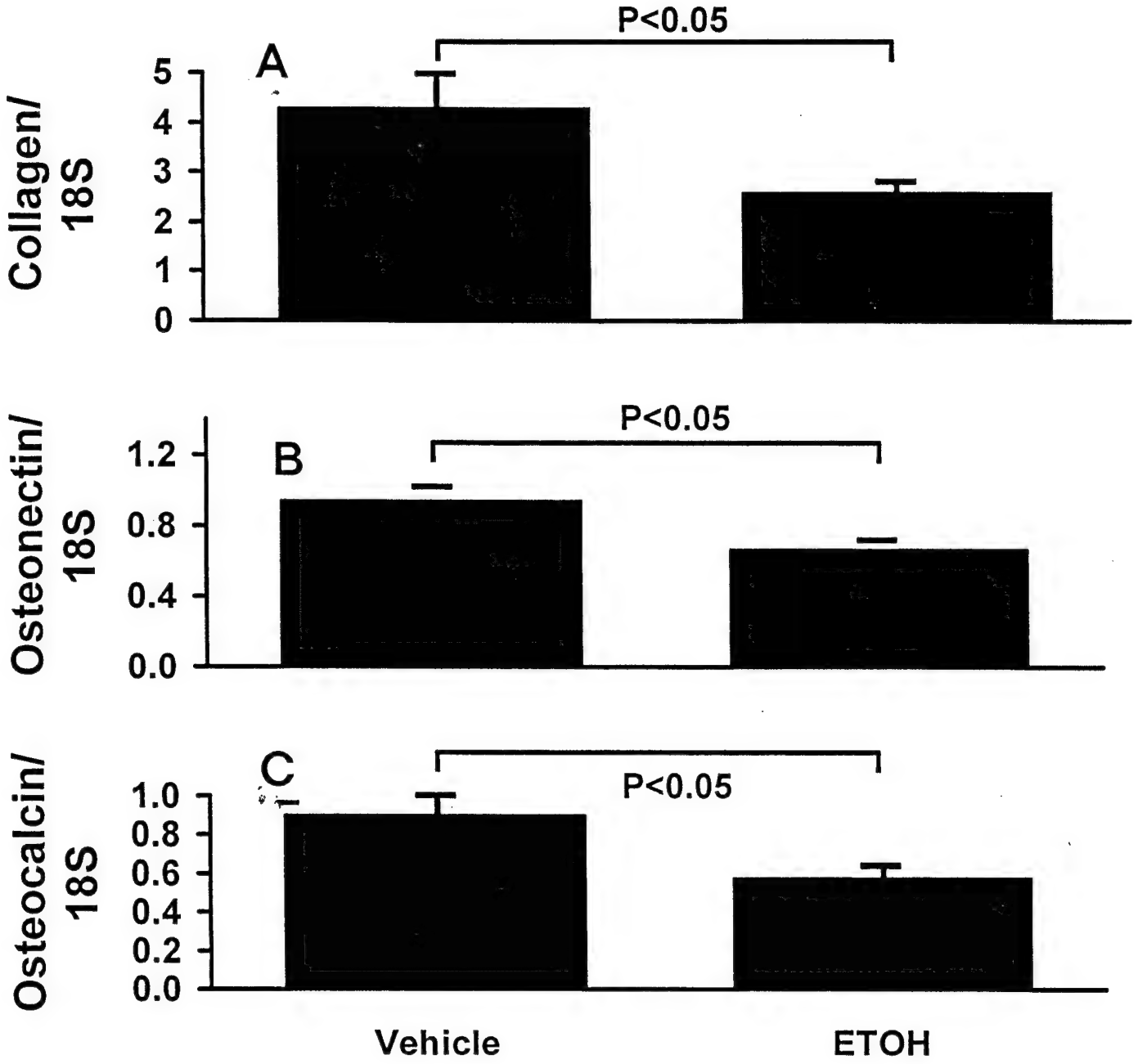

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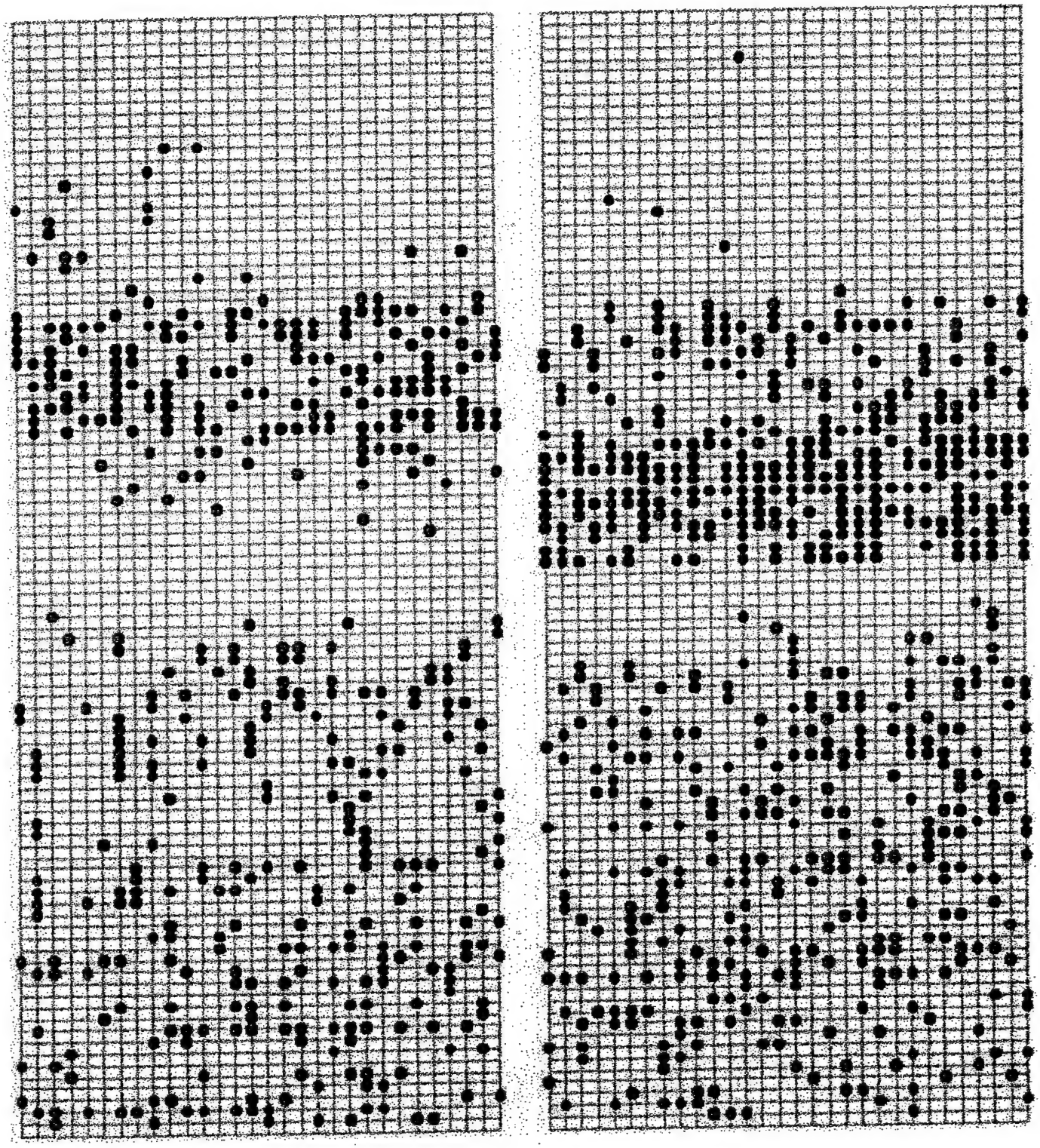

Appendix 3

ORGAN-SPECIFIC EFFECTS OF ALCOHOL ON GENE EXPRESSION

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ABSTRACT

BACKGROUND

Alcohol abuse can result in pathological changes in numerous organs. It is not clear whether common mechanisms underlie the diverse range in organ pathologies observed in alcoholics.

METHODS

The present cDNA microarray study was designed to investigate the effects of a brief exposure to ethanol (1 g/kg) on gene expression in 3 organs in sexually mature female rats.

RESULTS

Ethanol altered mRNA concentrations in an organ specific manner. The number of genes whose expression was altered varied greatly between organs with liver > bone > uterus. Only 1 of 5531 genes assayed was altered in all 3 organs.

CONCLUSIONS

The results demonstrate that the initial skeletal response to alcohol differs greatly from the response of liver and uterus, suggesting that organ-specific gene alterations play an important role in the development of alcohol-induced pathophysiology.

KEY WORDS: cDNA microarray, pathophysiology, alcohol abuse, gene expression, rat

RUNNING HEAD: Organ Specific Effects of Alcohol

INTRODUCTION

Chronic alcohol abuse results in a bewildering variety of pathological changes (Zakhari, 1991; Cook, 1998; Wilson and Pirola, 1997; Kaplowitz, 2000; Bikle et al., 1985). The organs affected include brain, liver, heart, bone, bone marrow, digestive system, and pancreas. Not all alcoholics develop organ pathology and the ones who do usually develop serious problems in only one organ.

Drinking is rarely likely to have a direct lethal effect on cells. It is more likely that ethanol disturbs cell signaling and as a consequence disrupts normal function. There has been great interest in the signaling pathways that are disturbed by alcohol and several have been identified, including membrane receptor-mediated stimulation of second messengers, phospholipase C signaling, serine/threonine protein kinases, ion channels, cell adhesion, and gap junctional signaling (Hoek et al., 1988; Diamond and Gordon, 1997; Mochly-Rosen et al., 1988; Hoeket al., 1987; Slater et al., 1993; Constantinescu et al., 1999; Resnicoff et al., 1996; Miyakawa et al., 1997; Harris, 1999; Wilkemeyer et al., 2000; Abou Hashieh et al., 1996). However, it is possible that a common mechanism of action underlies these organ-specific actions of ethanol. According to this view, specific organ pathologies associated with chronic drinking are the result of additional co-morbidity factors. We tested the possibility that acute exposure to ethanol induces a similar initial response in liver, bone and uterus by analyzing isolated total cellular RNA samples using a cDNA microarray.

METHODS

The animal studies were performed following approval of the Institutional Animal Welfare Committee. Sexually mature Sprague Dawley female rats obtained from Harlan Sprague Dawley, Madison, WI, were administered either ethanol 1 g/kg body weight as a 40%

aqueous solution or water only as described (Turner et al., 1998). The rats were anesthetized 24 h later with ketamin HCL (120 mg/kg:xylazine (24 mg/kg) prior to decapitation. Total cellular RNA was isolated as described (Cavolina et al., 1997) using a modified organic solvent method (Chomcynski and Sacchi, 1987). Gene filter microarrays were performed using rat Gene Filter microarray release 1 (GF300) and software from Research Genetics (Huntsville, AL). Total RNA (1 μg) isolated from bone was used for cDNA synthesis. The RNA was pooled from 4 animals from each treatment group to reduce differences due to individual animal variation. The synthesis of cDNA, prehybridization, hybridization and washings were performed as described in the manufacturer's protocol (Research Genetics). The radioactive Gene Filters were exposed for 48 h on a phosphor screen to obtain the image of the gel using the Cyclone Storage Phosphor System (Packard Instrument Company, Meriden, CT). The phosphor image was analyzed using Pathways software (Research Genetics) to determine gene specific changes.

The microgene array data was analyzed in a manner similar to Feng, et al. with the exception that we utilized a slightly more stringent criteria (2.5 vs 2.0 fold change) for reporting gene differences (Feng et al., 2000). Northern analysis was performed for selected genes included on the microgene array (Turner et al., 1998). The results verify that the mRNAs were expressed and that comparable changes following alcohol treatment were observed. An additional control consisted of repetitive hybridization of total cellular RNA pooled from control rats to a microgene array filter. Subtrative hybridization as performed for the control and alcohol-treated rats resulted in no detected gene changes.

RESULTS

Time course studies (Turner et al., 1998) revealed that administration of ethanol (1 mg/kg) results in peak blood alcohol level of ~0.1% 1 h later. Blood alcohol to below the

detection limit within 4 h. As shown in Figure 1, ethanol resulted in organ specific changes in gene expression. Of the genes evaluated (Table 1), ethanol resulted in changes (>2.5-fold increases or decreases) in steady-state mRNA levels for 54 genes in uterus, 497 in proximal tibial metaphysis and 3175 in liver (a complete list of the gene changes in the 3 organs can be obtained upon request [turner.russell@mayo.edu]). These results demonstrate that alcohol results in profound organ-specific differences in the number of genes whose expression is altered. The genes that changed included representatives of classes of peptides involved in virtually every aspect of cell function, including infrastructure, extracellular matrix, metabolism, and cell signaling. This finding suggests that even acute exposure to relatively modest concentrations of ethanol influences multiple signaling pathways.

None of the 28 named genes whose expression was altered by alcohol in uterus corresponded to alcohol-regulated genes in bone but all of these genes were also altered in liver. Expression levels of three of the expressed sequence tags (ESTs) were altered by alcohol in uterus and bone. In bone, 45 of the 134 named genes and 50 of the 124 ESTs whose expression was altered by alcohol were also regulated in liver.

The direction of the changes provides an additional indication of organ specificity. All 54 shared genes changed in the same direction in uterus and liver (Table 2), strongly suggesting a common pathway accessed by alcohol. However, comparing the 169 genes shared between bone and liver reveals only 66 that were regulated in the same direction, which suggests activation of different signaling pathways. This conclusion is further supported by the observation that of 5531 genes assayed, only one was altered by alcohol in all 3 organs and the direction of change of this gene differed between bone and the other two organs.

DISCUSSION

Ethanol induces a variety of pathophysiological changes in the uterus in non-pregnant as well as pregnant laboratory animals, including alterations in the methylation of membrane phospholipids, increased oxygen tension, impaired glucose homeostasis, altered spontaneous motility, prostaglandin production, triglyceride metabolism and tissue atrophy (Murdoch and Edwards, 1992; Mitchell and Van Kainen, 1992; Murdoch and Simm, 1992; Chaud et al., 1991; Murdoch, 1987). Many of these changes are believed to be indirect. Our results suggest that the uterine response to alcohol is associated with a limited number of genes and that these changes in gene expression share a common pathway with a subgroup of changes occurring in the liver.

Alcoholic liver disease is characterized by progressively severe changes. The initial pathology of fatty liver, which is reversible, is followed by fibrosis which may be reversible and cirrhosis which is irreversible (Lieber et al., 1975; Kaplowitz and Tsukamoto, 1996; Kaplowitz, 2000). Although acute administration of alcohol resulted in dramatic changes in gene expression in rat liver, even chronic exposure to high concentrations of alcohol is insufficient to induce irreversible alcoholic liver disease in the animal model (Porta et al., 1965; Tsukamoto and French, 1993). Co-morbidity factors such as dietary iron, estrogen and endotoxin appear to be needed (Thurman, 1998; Tsukamoto et al., 1995; Iimuro et al., 1997; Enomoto et al., 1999). Gene microarrays should provide a useful tool for investigating the interactions between these factors and alcohol.

Alcohol abuse disturbs bone and mineral homeostasis and is an important risk factor for osteoporosis (Spencer et al., 1986; Bikle et al., 1993; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Pumarino et al., 1996; Schnitzler and Solomon, 1984). Even small amounts of alcohol suppress bone formation (Bikle et al., 1985; Gonzalez-Calvin et al., 1993; Schnitzler and Solomon, 1984; Labib et al., 1989; Laitinen et al., 1991; Laitinen et al., 1994;

Nielsen et al., 1990; Rico et al., 1987). The rat skeleton is similarly sensitive to alcohol and the rat model duplicates human pathology with a high degree of fidelity (Turner et al., 1998; Peng et al., 1972; Peng et al., 1988; Turner et al., 1988; Turner et al., 1991; Wezeman et al., 1999; Turner et al., 1987; Dyer et al., 1998; Hogan et al., 1997; Sampson et al., 1998; Sampson et al., 1997; Sampson et al., 1999; Turner et al., 1998). The results of the current study indicate that the skeletal effects of moderate alcohol consumption on bone metabolism are associated with changes in the expression of a large number of genes. Furthermore, the pattern of changes differs considerably from liver. The regulation of mRNA levels for collagen is especially noteworthy because gene expression occurs in opposite directions. Excess collagen production occurs in the fibrotic liver whereas alcohol-induced osteoporosis is due, at least in part, to decreased collagen synthesis by osteoblasts (Bikle et al., 1985; Bikle et al., 1993; Turner et al., 1998; Nieto et al., 1999).

In summary, we have shown dramatic organ differences in the numbers of genes whose expression is altered by brief exposure to alcohol. Minimal overlap in gene changes between bone and uterus and a preponderance of changes in the opposite direction for bone and liver suggest these 3 organs exhibit important organ-specific differences in signaling pathways disturbed by alcohol. However, the similarity of the changes between liver and uterus provides strong evidence for conservation of a limited number of mechanisms of action between organs.

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Table 1: Organ-Specific Effects of Alcohol on Gene Expression			
Organ(s)	Named Genes (# changed)	ESTs (# changed)	Total Genes (# changed)
Uterus	28[1]	26	54
Tibia	134	363	497
Liver	1237	1938	3175
Uterus & Tibia	0	2/3[2]	2/3
Uterus & Liver	28/28	14/14	42/42
Tibia & Liver	16/45	50/124	66/169
All organs	0	0/1	0/1

[1]Number of genes which mRNA levels differ from control by $\geq$ 2.5 fold from a total of 5531 genes assayed.

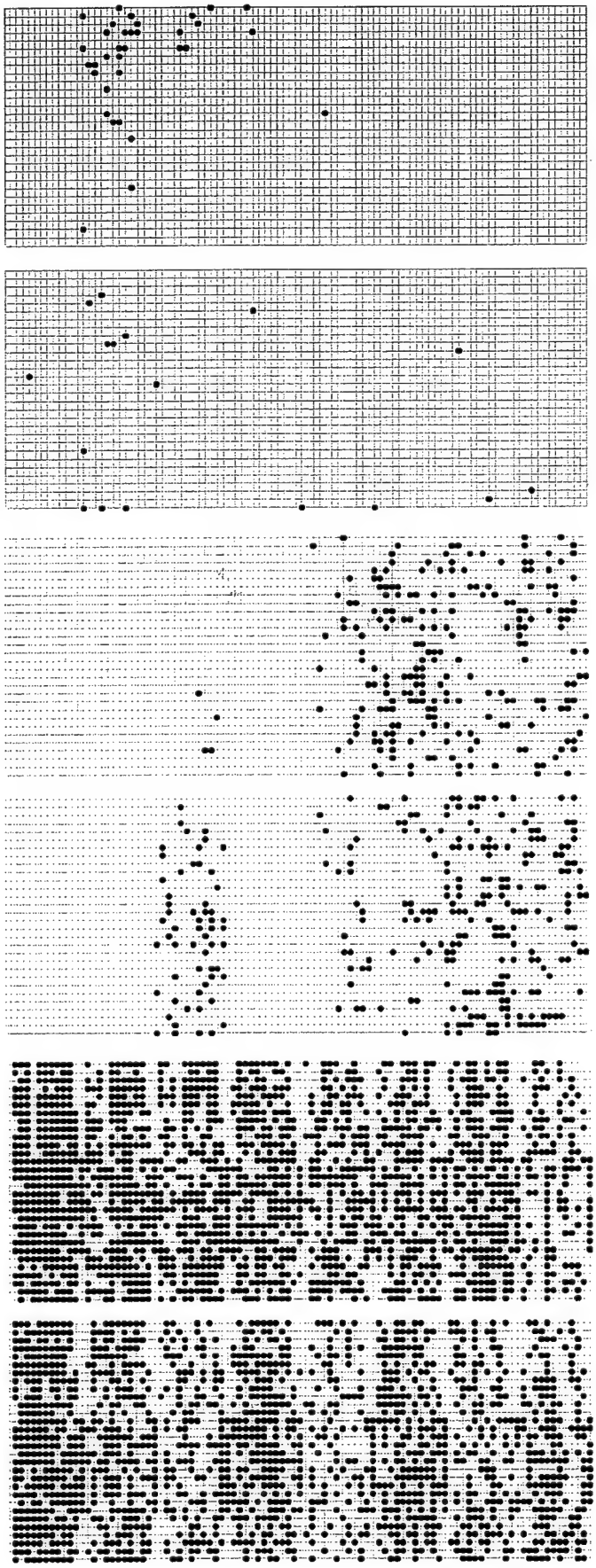
[2]x/y where x = number of genes where mRNA levels change in same direction and y = total number of genes in common regulated by alcohol.

Table 2: Named Genes Regulated by Ethanol in Uterus and Liver		
Gene	Uterus (fold change)	Liver (fold change)
Keratin (K5)	-2.6[1]	-3.3
RGS8	-2.7	-3.8
Calcium-independent alpha-latrotoxin receptor	-2.5	-2.8
Beta-tubulin T beta 15	-2.9	-2.9
Syntaxin binding protein Munc18-2 mRNA	-2.7	-3.1
Pancreatic phospholipase A-2	-2.9	-4.3
Proteasomal ATPase (Tat-binding protein 7)	-2.6	-2.9
Transcription factor USF-1	-2.5	-3.2
cdc25A	-2.6	-2.9
PMF31	-2.7	-3.1
Alternatively spliced aggrecan, large aggregating cartilage proteoglycan core protein	-2.6	-4.2
RB13-6 antigen	-2.5	-3.4
Iron-responsive element-binding protein	-2.5	-4.2
190 kDa ankyrin isoform	-2.8	-2.9
120 Kd lysosomal membrane glycoprotein	-2.5	-4.1
Farnesyltransferase beta subunit	-2.6	-3.8
Cortactin isoform B	-3.0	-4.2
BTG3 mRNA	-2.8	-4.6
Mitochondrial 3-2trans-enoyl-CoA isomerase	-2.7	-3.7
Oxidosqualene cyclase mRNA	-2.7	-3.6
Spermatid protein RSP29	-2.6	-3.5
RNB6	-2.6	-3.0
Zinc finger transcriptional activator (NGFI-C)	-2.8	-3.3
Transition protein 2 (TP2)	-2.9	-3.2
Cytochrome P450 2D18	-2.8	-3.1
P2X5 protein	-2.5	-3.3
Delta-aminolevulinate synthase	-2.6	-4.3
RAB10	-2.5	-3.3
[1]Values are fold change in steady-state mRNA levels compared to rats given water only.		

Table 3: Named Genes Regulated by Ethanol in Liver and Bone		
Gene	Liver (fold change)	Bone (fold change)
Alpha-lactalbumin (alpha-LA)	-5.9[1]	3.2
Brain acyl-CoA synthetase II	-8.6	4.1
Complex polypeptide 1 (Tcp-1)	-5.9	4.7
GTP cyclohydrolase I	-6.6	3.3
Carboxylesterase precursor	-6.8	3.1
Gamma subunit of sodium potassium ATPase	-6.6	3.9
F1-ATPase alpha subunit	2.8	-7.7
Farnesyltransferase alpha subunit peptide	-4.4	3.0
Equilbrative nitrobenzylthioninosine-insensitive nucleoside transporter	-9.6	4.7
CD30	3.6	-9.3
G protein gamma-5 subunit	-7.8	4.3
Bone morphogenetic protein 2 related peptide	-5.5	3.2
Lamina-associated polypeptide 1C (LAPIC)	-8.8	2.9
Cardiac troponin T mRNA	-4.1	-4.1
Microvascular endothelial differentiation gene 1	-9.5	2.5
Rab3B protein	-4.8	2.5
Long-chain acyl-CoA synthetase	-5.5	3.0
Gephyrin	-5.9	2.8
MIFR	-3.7	3.0
Alpha 2,6-sialyltransferase	-9.9	3.3
250 kDa estrous-specific protein	-3.5	2.5
Retrovirus-related gag protein	-9.4	4.1
EGF-like growth factor	-7.5	2.6
Galanin receptor type 2 (GALR2)	-7.4	2.6
RET ligand 1 (RETL1)	-9.5	5.8
Nedd2/Ich-1 mRNA	-2.7	-2.5
Very-long-chain Acyl-CoA dehydrogenase	-7.4	-4.2
mRNA for ST1B1	-3.2	2.5
Vesicular GABA transporter (VGAT)	-3.3	-2.6
Peroxisome assembly factor-2	-3.1	2.6
Retinol binding protein (CRBP)	-3.2	-3.2
Proto-oncogene FYN (p59fyn)	-4.8	-2.8
Clathrin-associated protein 17 (AP17)	-8.0	-3.8
V-1 protein	-3.4	-5.4
Neurabin mRNA	-2.9	-4.4
Collagen alpha 1 type 1	-3.7	2.6
Intercellular calcium-binding protein (MRP8)	-4.4	-4.1
p-450, phenobarbital-inducible	3.2	5.8
AlphaB crystallin-related protein	-2.9	-2.7
Ferritin-H subunit	-3.9	2.5
Acyl-CoA synthetase	2.9	3.0
Alpha-globin mRNA	-6.9	2.6
Peptidylarginine deiminase	-3.4	-2.9
Mannose 6-phosphate/insulin-like growth factor II receptor	-3.0	2.6
Ribosomal protein S10	-3.4	-2.8
[1]Values are fold change in steady-state mRNA levels compared to rats given water only.		

FIGURE LEGEND

Figure 1: cDNA microarrays used to detect genes regulated by alcohol in uterus (top), proximal tibial metaphysis (middle), and liver (bottom). The microarrays were sequentially hybridized to total RNA from control rats and alcohol treated rats. Only genes expressing differences in mRNA levels of $\geq$ 2.5 fold are shown. Red spots indicate genes with increased expression following alcohol whereas blue indicates decreased expression.



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EFFECTS OF PARATHYROID HORMONE ON BONE FORMATION IN ALCOHOLIC RATS

Running Title: Alcohol and PTH

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ABSTRACT

Alcohol abuse is an important risk factor for osteoporosis. The present study was designed to examine the effects of chronic high-dose alcohol consumption on the skeletal response to parathyroid hormone (PTH), a hormone that is being investigated for treatment of osteoporosis. Ethanol was administered in the diet of female rats (35% caloric intake) for two weeks. PTH (80 μg/kg/d) was administered s.c. during the second week of the study. Alcohol resulted in a transient reduction in steady-state mRNA levels for several bone matrix proteins, including type 1 collagen, osteocalcin, and osteonectin, compared to time-matched rats fed a control diet. As expected, alcohol decreased and PTH increased histological indices of bone formation. Additionally, two-way ANOVA demonstrated that alcohol antagonized PTH-induced bone formation. In spite of antagonism, bone formation and mRNA levels for bone matrix proteins in alcohol-fed rats treated with PTH greatly exceeded the values in rats fed the control diet. These results suggest PTH treatment may be a useful intervention to treat alcohol-induced bone loss.

Key Words: alcoholism, osteoblasts, osteoclasts, ethanol, osteopenia, bone loss

INTRODUCTION

Alcoholics often have radiographic evidence of osteopenia, a greatly reduced bone mineral density and reduced histological and biochemical indices of bone formation (1-15). There is also evidence that alcoholics are at a greater fracture risk than healthy individuals (16,17). Alcohol-induced osteoporosis differs from postmenopausal bone loss in that the pronounced increase in bone turnover that follows the menopause is not observed in alcoholics (18,19). The principal mechanism for alcohol-induced bone loss appears to be reduced bone formation (3,5,7,11-13); indices of bone resorption may be increased, decreased or unchanged (2,4-7,14,20,21). The net reduction in bone formation appears to result in a negative remodeling balance and ultimately to osteopenia.

At present, there is no specific intervention to treat alcoholics who have osteoporosis, but a rat model for the skeletal effects of alcohol abuse has been established to investigate possible therapies. Rats fed ethanol at a rate (adjusted for the difference in body mass) comparable to alcoholics develop abnormalities in bone and mineral homeostasis similar to alcoholics (22-28). These abnormalities include osteopenia and a pronounced inhibition of bone formation (24-28).

Parathyroid hormone (PTH) is under investigation for treatment of osteoporosis. Pulsatile administration of PTH increases bone formation and bone mass in humans and laboratory animals. The principle purpose of the present study was to evaluate the efficacy of PTH as a therapy to reverse the inhibitory effects of alcohol abuse on bone formation in the rat model.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Welfare Committee.

Six-month-old female Sprague Dawley rats (Harlan, Sprague Dawley, Indianapolis, IN) weighing an average of 294 ± 2 g (mean ± SE) were randomized and assigned to one of six groups (n = 10 rats/group). Rats were individually housed in a temperature- and humidity-controlled animal facility on a 12-hour light/dark cycle. All animals were acclimated to a modified Lieber-DeCarli liquid diet (BioServe, Frenchtown, NJ) for one week. The three alcohol-supplemented groups were subsequently fed, ad libitum, a liquid diet containing increasing concentrations of ethanol (95% v/v) until receiving 35% of total caloric intake as alcohol at the end of the first treatment week (29). This dose of alcohol results in blood alcohol levels and changes in bone and mineral metabolism similar to alcoholics (24-29). Rats were not given water. The three control diet groups did not receive ethanol and were fed the same liquid diet isocalorically supplemented with maltose/dextran, as per the manufacturer's instructions. All animal weights were recorded on the first day of study (day 1), on the first day of treatment with PTH (day 8) and on the day of necropsy (day 15). The 24-hour consumptions were recorded daily and the rats fed the control diet were pair-fed to the mean consumption of the alcohol-treated animals.

A schematic of the experimental design is shown in Figure 1. On the first day of ethanol treatment, all rats were injected with a 20 mg/kg BW dose of a bone fluorochrome, tetracycline-hydrochloride (Sigma, St. Louis, MO), perivascularly at the base of the tail. All rats were injected 7 days later with another bone fluorochrome, calcein (20 mg/kg BW; Sigma). One group of rats fed alcohol and one group of rats fed the control diet were sacrificed on day 8 to provide baseline measurements.

Starting on day 8, two groups of rats (one fed the control diet and one fed alcohol) were administered human PTH (1-34) daily (80 μg/kg) sc, as described (30). Also, starting on day 8,

the remaining two groups of rats (one control diet and one fed alcohol) were given carrier only. All remaining rats were given the fluorochrome demeclocyline (20 mg/kg BW; Sigma) on day 14 and sacrificed on day 15.

Rats were anesthetized using ketamine (100 mg/ml) and xylazine (100 mg/ml) and sacrificed by decapitation. Tibiae were defleshed and either frozen in liquid nitrogen and stored at -80°C prior to RNA analysis or fixed by immersion in 70% ethanol for bone histomorphometry. Uteri were harvested and wet weights recorded.

Bone Histomorphometry

Tibiae were dehydrated by immersion in a series of increasing concentrations of ethanol, and embedded without demineralization in a methylmethacrylate mixture (methylmethacrylate:2-hydroxyethyl-methacrylate 12.5:1). Longitudinal sections were taken from the middle of the proximal tibia using a Reichert-Jung microtome (5 μm thick) and permanently mounted on slides for histomorphometric measurements. Cancellous bone measurements were performed in a standard sampling site located on the long axis of the bone, one millimeter from the epiphyseal growth plate at its most distal point. This site is distal to the primary spongiosa, within the secondary spongiosa, and extends bilaterally to exclude the endocortical edges. The sampling site encompassed a tissue area of 2.88 mm^2.

All histomorphometric measurements were carried out with a semi-automatic image-analysis system, which consists of a Compaq computer interfaced with a microscope and image analysis software (OsteoMetrics, Inc., Atlanta, GA). Skeletal indices were measured by registering the movement of a digitizing mouse across a graphics tablet as a tracing was superimposed on an image of the section displayed on a video screen. The computer software recorded lengths and calculated the enclosed areas. The following histomorphometric indices

were determined as described (29): cancellous bone area (BA), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), mineralizing perimeter (L.Pm), mineral apposition rate (MAR), and bone formation rate (BFR). Measurements were performed in accordance with standardized nomenclature and methods (31).

Northern Hybridizations

Total RNA was isolated from tibial metaphyses for Northern analysis of steady-state mRNA levels for selective bone matrix proteins. Metaphyses from frozen tibiae were individually crushed into powder using a Spex freezer mill (Edison, NJ) and total cellular RNA was extracted and isolated with a modified organic solvent method as described (32). Ten μg of total RNA from each sample was denatured in 1M glyoxal, 50% dimethylsulfoxide 0.01 M NaH_2PO_4 at 52°C, separated by electrophoresis on a 1% agarose gel, transferred by capillary action in 20X SSC to nylon membranes (Amersham Hybond nylon membrane, Arlington Heights, IL) and crosslinked using a UV source (Stratagen, LaJolla, CA) before hybridization procedures. Membranes were prehybridized for 1-2 hrs at 65°C in a Rapid-Hyb Buffer (Amersham). Hybridization was carried out for 80 minutes in a buffer containing the above ingredients in addition to a minimum of $4x10^6$ cpm/ml ^{32}P-labeled cDNA for the following bone matrix proteins: prepro-α (I) subunit of type I collagen (collagen), osteocalcin and osteonectin. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and by hybridization with ^{32}P-labeled cDNA for 18S ribosomal RNA. Representative Northern blots of bone extracts from ethanol and from PTH-treated rats are published (30,32).

cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit (Amersham, Arlington Heights, IL). The membranes were washed for 30 minutes at RT in 3X SSC and for 15 minutes in 1X SSC at 43-65 °C.

The mRNA bands on the Northern blots were quantitated by densitometric scanning (Molecular Dynamics Phosphor Imager, Sunnyvale, CA) and values normalized to 18S ribosomal RNA. Representative photographs of phosphor images of Northern blots for type 1 collagen, osteocalcin and osteonectin are published (32).

Statistical Analysis

Values are expressed as mean ± standard error. Group comparisons between the alcohol-treated and non-alcohol fed groups following the first week of treatment were determined by Student's t-test. The respective effects of ethanol and PTH were analyzed by two-way analysis of variance. Significance was established at p values ≤ 0.05.

RESULTS

Body weights and uterine weight are shown in Table 1. Neither alcohol nor PTH had an effect on body weight or uterine weight.

The effects of one week of dietary alcohol on bone histomorphometry ares shown in Table 2. Alcohol had no significant effects but there was a consistent tendency toward reduced dynamic measurements, especially LS/BS, BFR/TV, BFR/BS, and BFR/BV.

The effects of one week of dietary alcohol on steady-state mRNA levels for bone matrix proteins are shown in Figure 2. Alcohol resulted in significant decreases in mRNA levels for type 1 collagen, osteonectin, and osteocalcin.

Table 3 shows the effects of two weeks of dietary alcohol with and without PTH treatment on bone histomorphometry. Alcohol decreased and PTH treatment increased several

indices of bone formation, including LS/BS, BFR/BS, BFR/BV, and BFR/TV. Neither treatment had a significant effect on MAR, although there was a strong tendency for alcohol to reduce this measurement. Alcohol significantly reduced but did not prevent the stimulatory effect of PTH on bone formation.

The effects of PTH treatment with and without alcohol on steady-state mRNA levels for bone matrix proteins are shown in Table 4. PTH increased mRNA levels for type 1 collagen, osteocalcin and osteonectin. Alcohol had no significant effects on mRNA levels for the three bone matrix proteins after two weeks of treatment. However, it tended to decrease the mRNA levels for type 1 collagen and osteocalcin and tended to decrease the stimulatory effects of PTH on mRNA levels for all three bone matrix proteins.

DISCUSSION

Alcohol abuse leads to decreased bone formation in humans (3,5,7,11-13) and experimental animals (24-28). The present studies demonstrate that the pronounced inhibitory effects of alcohol on bone formation in adult rats can be reversed by PTH, even while rats continue an alcohol intake comparable to alcoholics.

The inhibitory effect of alcohol consumption on bone formation occurred within two weeks of initiating treatment. The decreased mRNA levels for bone matrix proteins and strong tendency toward reduced dynamic bone measurements observed in the alcohol consuming baseline group suggest that the inhibitory effects of alcohol on bone formation were established prior to initiation of PTH treatment. The failure to detect significant changes in dynamic bone histomorphometry in the alcohol-treated rats after one week is not unexpected. The fluorochrome based measurements would underestimate an inhibitory response because L.Pm, MAR and BFR are calculated using two fluorochrome labels, one of which was administered at

the time treatment was started, at which time bone formation would not differ from the controls. Although the present study was only two weeks in duration, we have shown that bone formation is also suppressed in adult rats continuously fed a diet in which ethanol contributes 35% of the caloric intake after 2 and 4 months (Turner, unpublished data).

The inhibitory effects of alcohol on steady-state mRNA levels for bone matrix proteins were transient; mRNA levels returned toward normal after two weeks of alcohol consumption. This finding was, not surprisingly, based on the results of time course studies showing that the effects of alcohol on gene expression can change dramatically over short intervals (32). We have also shown that chronic consumption of alcohol can suppress bone formation and cause bone loss without a continuous reduction in mRNA levels for bone matrix proteins (Turner, unpublished data). Taken together, these findings suggest, but do not prove, that alcohol-induced inhibition of bone formation may be due in part to post-transcriptional alterations. Alternatively, the mRNA levels for bone matrix proteins may depend upon the interval between feeding and sacrifice.

PTH resulted in histomorphometric changes consistent with those reported in published studies, including increases in mineralizing perimeter and bone formation rate (29,33,34). The large increases in mRNA levels for bone matrix proteins following PTH-treatment have also been reported (29). The lack of an effect of PTH on bone area and architecture was anticipated because of the short duration of the study (29,33,34).

PTH increases bone formation in rats by increasing osteoblast number and to a lesser extent activity (29,33,34). In the current study, LS was measured as an index of osteoblast number, and MAR was calculated as an index of osteoblast activity (35). As previously shown,

the increase in LS was largely responsible for the PTH-induced increase in bone formation (29,33,34).

Alcohol consumption inhibited bone formation by decreasing LS and had little effect on MAR. These findings are in good agreement with prior rat (24-28) and human studies (2-8).

Prolonged exposure to high concentrations of alcohol inhibits proliferation of cultured osteosarcoma cells (36,37), immortalized cells derived from the osteoblast lineage (38), and primary osteoblastic cell cultures from chick calvaria (39). Alcohol, however, had little effect on differentiated osteoblasts suggesting that the reduction in LS in rats consuming alcohol may be due in part to decreased cell proliferation (36-38). On the other hand, failure to detect significant reductions in mRNA levels for bone matrix proteins after two weeks of treatment is not consistent with this mechanism. We have no explanation for this apparent discrepancy but have made similar observations in other experiments.

In the present study, chronic alcohol consumption blunted the anabolic effects of PTH on bone. Others have reported that alcohol reduces PTH levels in humans (40) and antagonizes the hypercalcemic effects of PTH in rats and dogs (22,23). Thus, reduced circulating levels of PTH and/or end organ resistance to the hormone may contribute to alcohol-induced bone loss. In spite of the observed antagonism, PTH-treatment increased bone formation in the alcohol-treated rats to values which greatly exceeded those observed in rats fed the control diet. Furthermore, PTH treatment reversed the reduction in LS observed following chronic alcohol consumption. These findings suggest that PTH treatment may be useful as an intervention to reverse alcohol-induced osteopenia. However, additional long-term studies will be necessary to demonstrate that PTH treatment is capable of restoring bone mass in rats with alcohol-induced osteopenia.

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Table 1: Effects of Alcohol and PTH on Body Weight and Uterine Weight		
Group	Final Body Weight (g)	Uterine Weight (g)
Baseline		
Control diet	305±5	865±83
Alcohol diet	295±6	657±64
t-test		
Effect of Alcohol	NS	NS
Treatment		
Control Diet		
Vehicle	307±5	558±48
PTH	305±5	637±69
Alcohol Diet		
Vehicle	280±5	509±34
PTH	285±5	496±49
Two-way ANOVA		
Effect of Alcohol	NS (.09)	NS
Effect of PTH	NS	NS
Interaction	NS	NS
Values are mean ± SE; N = 10.		

Table 2: Effects of Alcohol on Baseline Cancellous Bone Histomorphometry After One Week Treatment

Measurement	Control Diet	Alcohol Diet	P Value (t-test)
BV/TV (%)	25.1±2.9	29.2±1.9	NS
Tb.Th (μm)	56.8±3.2	59.7±3.1	NS
Tb.N (mm^{-1})	4.35±0.32	4.88±0.19	NS
Tb.Sp (μm)	180±20	147±9	NS
LS/BS (%)	2.30±1.6	1.40±0.60	NS
BFR/BS ($\mu m^3/\mu m^2/d$)	0.022±0.007	0.011±0.006	NS
BFR/BV (%/d)	0.080±0.025	0.033±0.016	NS
BFR/TV (%/d)	0.018±0.004	0.011±0.006	NS
MAR (μm/d)	0.94±0.05	0.78±0.10	NS

Values are mean ± SE; N = 7-8. NS is $P > 0.05$.

Bone volume (BV), tissue volume (TV), labeled surface (LS), bone surface (BS), mineral apposition rate (MAR), bone formation rate (BFR), trabecular (Tb), thickness (Th), number (N), separation (Sp).

Table 3: Effect of PTH on Bone Histomorphometry in Rats Fed Alcohol For Two Weeks

Measurement	Group				Two-Way ANOVA		
	Control Diet & Vehicle	Alcohol Diet + Vehicle	Control Diet & PTH	Alcohol Diet & PTH	Alcohol	PTH	Interaction
BV/TV (%)	22.2±1.9	29.4±2.2	27.1±1.9	24.9±1.5	NS	NS	NS
Tb.Th (μm)	55.2±2.2	60.0±2.9	64.7±2.4	59.8±1.8	NS	NS (.06)	NS
Tb.Sp (μm)	204.6±18.7	165.9±12.8	182.4±15.7	186.7±14.8	NS	NS	NS
Tb.N(mm^{-1})	4.0±.2	12.7±8.4	4.2±.2	4.1±.2	NS	NS	NS
LS/BS (%)	4.8±1.3	1.0±.4	30.1±3.1	11.7±1.8	.0001	.0001	.0007
MAR (μm/d)	1.09±.06	.88±.02	1.15±.04	.98±.03	NS (.06)	NS	NS
BFR/BS ($\mu m^3/\mu m^2$/d)	.05±.02	.01±.005	.35±.04	.12±.02	.0001	.0001	.0009
BFR/BV (%/d)	.19±.05	.04±.01	1.10±.12	.40±.07	.0001	.0001	.001
BFR/TV (%/d)	.04±.01	.01±.004	.29±.04	.08±.009	.0001	.0001	.0005

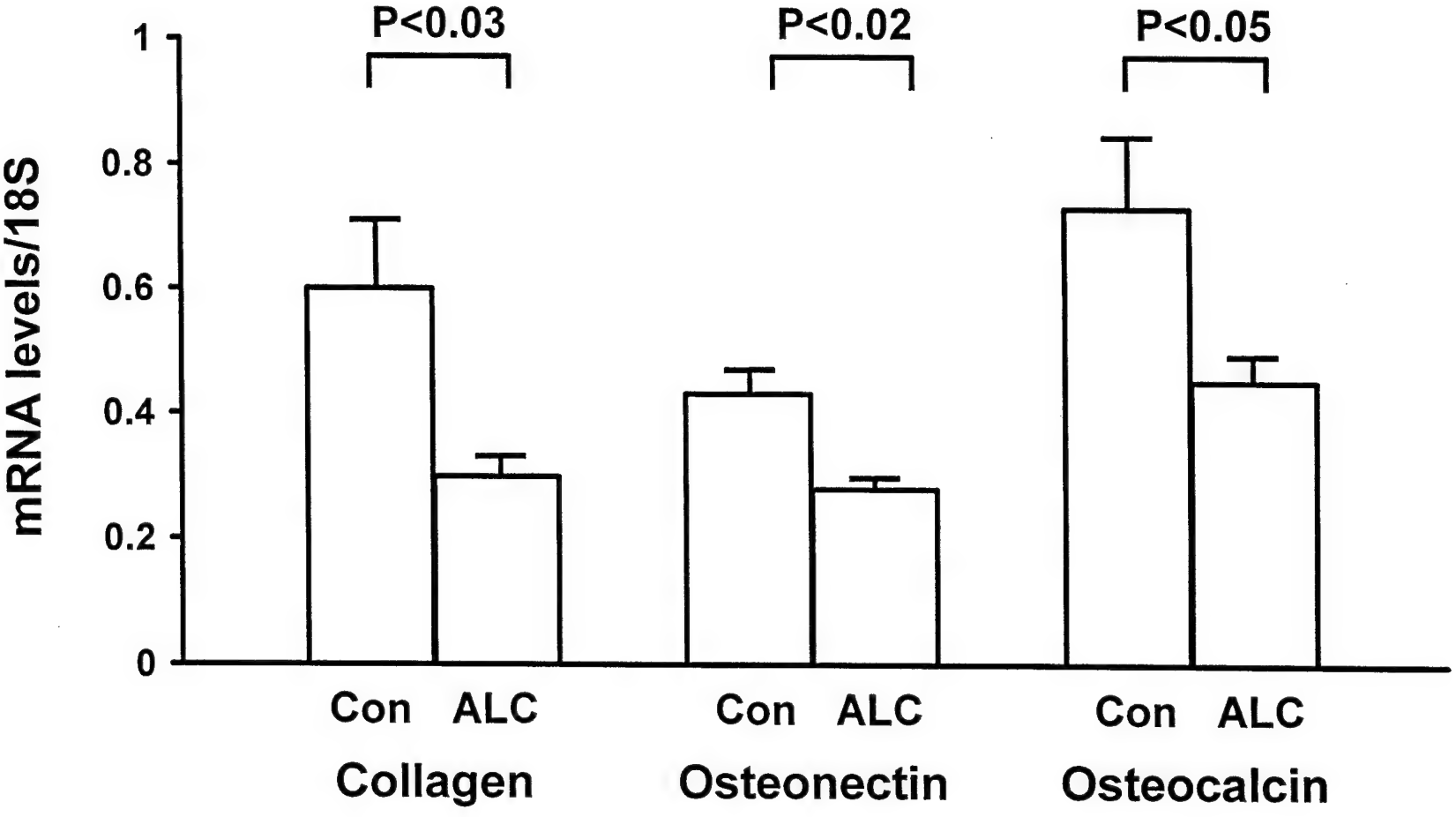
Bone volume/tissue volume (BV/TV), Trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), Trabecular number (Tb.N), label surface/bone surface (LS/BS), bone formation rate/bone surface (BFR/BS), mineral apposition rate (MAR), bone formation rate/bone volume (BFR/BV), bone formation rate/tissue volume (BFR/TV).

Table 4: Effect of PTH on Steady-State mRNA Levels for Bone Matrix Proteins in Rats Fed Alcohol for Two Weeks			
Group	Type 1 Collagen/18S	Osteonectin/18S	Osteocalcin/18S
Control diet	10.4±2.9	3.7±0.9	12.0±2.9
Alcohol diet	9.1±1.7	3.9±0.4	11.6±0.7
Control diet & PTH treated	53.8±4.6	16.3±2.1	54.5±3.6
Alcohol diet & PTH treated	35.3±7.1	11.1±1.2	39.1±9.1
Two Way ANOVA			
Effect of Alcohol	NS (p=0.05)	NS (p=0.08)	NS
Effect of PTH	P < 0.0001	P < 0.0001	P < 0.0001
Interaction	NS (p = 0.09)	NS (p = 0.06)	NS
Values are mean ± SE; N = 4.			

FIGURE LEGENDS

Figure 1: A schematic showing the experimental design. Alcohol treatment was started on day 0 and PTH treatment was started on day 8. Fluorochrome labels (⇑) were administered on day 0, 7, and 14. The baseline control groups were sacrificed on day 8 and the remaining groups on day 15.

Figure 2: The effects of a one week treatment with alcohol on steady-state mRNA levels for bone matrix proteins. Values are mean ± SE; N = 4. Alcohol diet (Alc), control diet (Con).


P<0.03
P<0.02
P<0.05
mRNA levels/18S
1
0.8
0.6
0.4
0.2
0
Con
ALC
Collagen
Con
ALC
Osteonectin
Con
ALC
Osteocalcin

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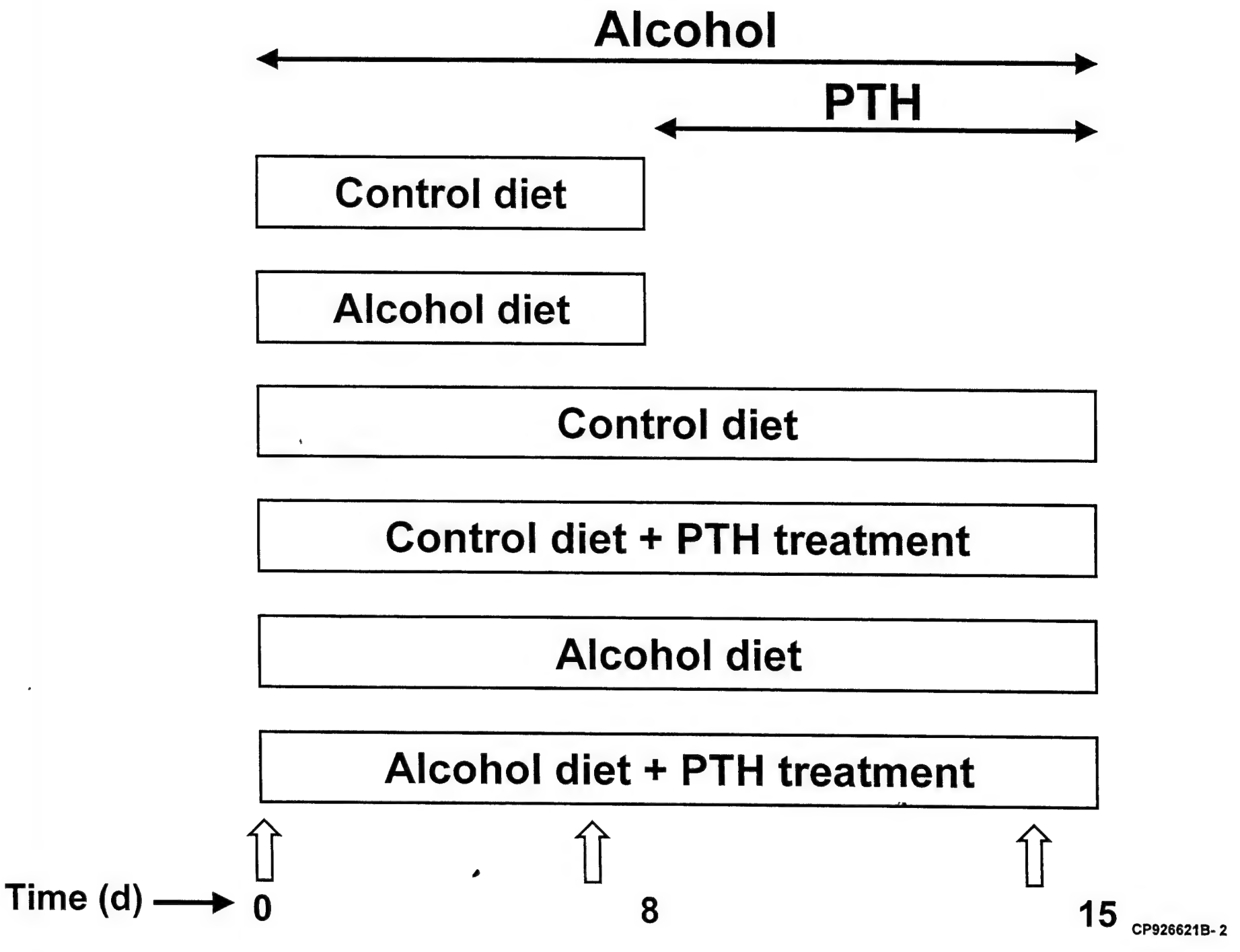
Alcohol
PTH
Control diet
Alcohol diet
Control diet
Control diet + PTH treatment
Alcohol diet
Alcohol diet + PTH treatment
Time (d)
0
8
15
CP926621B-2

The Dose Response Effects of Ethanol on Human Fetal Osteoblastic Cell Line

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Running Tittle: Ethanol action on fetal human osteoblast

Key words: Alcohol abuse, bone formation, osteoporosis, bone fractures

ABSTRACT

Alcohol is a risk factor for the development of osteoporosis, especially in men. Chronic alcohol abuse decreases bone mass which contributes to the increased incidence of fractures. To better understand the mechanism of action of ethanol on bone metabolism, we have studied the dose response effects of ethanol on conditionally immortalized human fetal osteoblasts (hFOB) in culture. Ethanol treatment had no significant effects on osteoblast number after 1 or 7 days. Ethanol treatment did not reduce type I collagen protein levels at either time point at any dose but slightly reduced alkaline phosphatase activity after 7 days. The mRNA levels for alkaline phosphatase, type I collagen and osteonectin were unaltered by 24hrs of ethanol treatment but a high dose (200 mM) reduced mRNA levels for the two bone matrix proteins after 7 days. Ethanol treatment led to dose-dependent increases in TGF-β1 mRNA levels and decreases in TGF-β2 mRNA levels. The concentration of ethanol in the medium decreased with time due to evaporation but there was little degradation due to metabolism. These results, which demonstrate that cultured osteoblasts are less sensitive than osteoblasts *in vivo*, suggest that the pronounced inhibitory effects of ethanol on bone formation are not due to direct cell toxicity.

INTRODUCTION

Chronic alcohol abuse is associated with pronounced detrimental effects on the musculoskeletal system. Numerous reports implicate alcohol as a major risk factor for osteoporosis, especially in men. Habitual alcohol abuse clearly results in bone loss while moderate intake of alcohol has been reported to have variable effects on bone mass, depending upon age and gender (1). However, the mechanism for the bone loss induced by alcohol is not understood. There is consensus that alcohol abuse results in decreased bone formation (2, 3). On the other hand, the effects of alcohol on bone resorption are less certain with a number of conflicting reports (4-9).

Alcohol results in dose dependent decreases in bone formation with dose rates comparable to alcoholics, leading to osteopenia (10-13). The molecular mechanism(s) that mediate this bone loss are poorly characterized. Some studies suggest that ethanol has a direct toxic effect on osteoblasts (14). The histological changes to bone are preceded by changes in mRNA levels for bone matrix proteins and cytokines (12). Rapid (within 6 hr) transient dose dependent changes in expression of matrix proteins in bone has been reported in the rat suggesting that exposure to ethanol results in reversible changes in osteoblast function. Farley *et al.,* have shown that chemicals, including ethanol, modify membrane fluidity and alter the response of bone cells to mitogens (15). This suggests that some of the actions of ethanol *in vivo* may be influenced by systemic factors, including gonadal hormones and cytokines. To better understand the mechanism of ethanol action on skeletal development, we have studied the direct effects of ethanol on immortalized human fetal osteoblast (hFOB) cells (16) in culture. In these studies, hFOB cells were exposed to ethanol and concentrations corresponding to blood alcohol levels relevant to

moderate drinking (10mM; 0.046% w/v) and chronic alcohol abuse (50 mM; 0.23% w/v), as well as concentrations incompatible with human life ($\geq$ 100 mM; 0.46% w/v).

MATERIALS AND METHODS

Cell Culture and Ethanol Treatment

The hFOB cell line that contains the temperature sensitive T antigen expression vector with neomycin resistance gene (16) was maintained at 34°C in phenol red-free DMEM/F12 containing 10% charcoal-stripped FBS and supplemented with geneticin (300μg/mL). The cells were plated into T-75 flasks at 1 X 10^6 cells per flask 24 hrs prior to ethanol treatment. The cells were treated with ethanol (10-500 mM) for 1 day and 7 days. During 7-day treatment the media were changed with fresh medium containing ethanol on day 4 and maintained for an additional 3 days. Cells were washed with PBS and harvested at the end of ethanol treatment for RNA analyses. The media was stored and used for type I collagen protein analyses.

Ethanol Assay

Ethanol content in media was determined using a kit as per the manufacturer's protocol (Sigma Chemical Company, St. Louis, MO).

RNA Isolation

Total cellular RNA was extracted and isolated using a modified organic solvent method and the RNA yields were determined spectrophotometrically at 260 nM (17).

Northern Blot Hybridization

Ten micrograms of each sample were denatured by incubation at 52°C in a solution of 1M glyoxal, 50% dimethyl sulfoxide, and 0.01 M NaH_2PO4, and then separated in a 1% agarose gel. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and hybridization with a [^{32}P]-labeled cDNA for 18S ribosomal RNA. RNA separated in agarose gels was transferred to an Amersham Hybond nylon membrane overnight via capillary action in 20 x standard saline citrate (SSC) (1xSSC=0.15 M NaCl and 0.015 M sodium citrate, pH7.0), sodium citrate buffer. The membranes were baked in a vacuum oven at 80°C for 2 hrs prior

to hybridization. Membranes were prehybridized for 2 hrs at 65°C in buffer containing 50% deionized formamide, 10% dextran sulfate, 5x SSC, 100 μg/ml of heat-denatured single-strand salmon sperm DNA and 2x Denhardts solution. Hybridization was conducted for 80 min in a buffer containing the above ingredients in addition to a minimum of $1x10^6$ cpm per mL [^{32}P]-labeled cDNA probe. Labeled cDNAs for alkaline phosphatase, osteocalcin and type I collagen were used as probes. cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit from Amersham (Arlington Heights, IL). Membranes were washed for 30 min at 45°C in 2xSSC and for 15 to 60 min in 0.1xSSC at 45°C. The resulting radioactive mRNA bands on the blots were quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and normalized to 18S rRNA.

The cDNA probes were: 1) Alkaline Phosphatase cDNA, a gift from Dr. Gideon Rodan (Merck, Sharp, and Dohme, West Point, PA; 2) Type I collagen cDNA probe obtained from Lofstrand Labs., Ltd. (18); 3) Osteonectin cDNA, a gift from Dr. G. Long (University of Vermont, Burlington, VT) (19); 4) cDNA for 18S rRNA was purchased from Ambion (Austin, TX).

RNase Protection Assay for Cytokines

We measured the mRNA concentrations of Transforming Growth Factor-beta (TGF-β) 1, 2, 3; Tumor Necrosis Factor (TNF) α and β; Interleukin (IL) 1 α, 1 β, 1Ra, 6, 10, 12 (p35 and p40); Interferon (IFN) β and γ and Lymphotoxin (LT) β by RNase Protection assays. Quantitation of protected RNA fragments was performed by PhosphorImager analyses and normalized to Glyceraldehyde 3-phosphate dehydrogenase and ribosomal structural protein L32.

Type I Collagen Protein Assay

The medium from ethanol treated hFOB cells were collected at the end of 24 hrs and 7 days of ethanol treatment, centrifuged to remove debris before used for type I procollagen assays. The type I procollagen assay, which measures the propeptide portion of the molecule, reflects the synthesis of the mature form of the protein and it was carried out using Prolagen-C kit as described by the manufacturer's protocol (Metra Biosystems, Mountainview, CA). The type I procollagen levels obtained were normalized to total protein concentrations which was determined by BCA protein assay (Pierce, Rockford, IL).

Alkaline Phosphatase Activity

The alkaline phosphatase activity was measured using a kit (Sigma Chemical Company, St. Louis, MO). The cells treated with ethanol were rinsed with phosphate-buffered saline, harvested and processed for determining the alkaline phoshatase activity. The activity was normalized to total cellular protein which was determined by BCA protein assay.

Statistical Analysis

In each experiment, 3-6 replicates of each treatment were measured. Unless indicated otherwise, the data represents the mean $\pm$ SE of three independent experiments. Significant differences between groups were determined by Fisher's Protected Least Significant Difference post hoc test for multiple group comparisons following detection of significance by 1-way ANOVA. Significance was considered at p values < 0.05. Dose response effects were analyzed by linear regression analysis.

RESULTS

Effect of Ethanol on Cell growth

The effect of ethanol treatment on human fetal osteoblast (hFOB) cell growth was determined after 1-day and 1-week treatment with 0, 50 and 200 mM concentrations of ethanol. The viable cell numbers were measured by trypan blue dye exclusion assay in a hemocytometer. There was no change in cell number after 1-day treatment (data not shown). However, after 1-week treatment there was an increase in cell number at 50mM and decrease in cell number at 200 mM concentrations of ethanol (Fig. 1). But these changes in cell number were not statistically significant.

Assay for Alcohol Levels

The amount of ethanol present in the media as a function of time after incubation at 34°C was determined in the presence and absence of cells (Fig. 2). The ethanol disappearance curves follow the same pattern in the presence and absence of cells. About 24% of the initial amount was still detectable after 4 days of incubation, the time at which replacement of new media containing ethanol was performed.

Effect of Ethanol on Bone Matrix Gene Expression in Cultured Human Osteoblasts

The dose (0-200 mM) effects of 24 hrs of ethanol treatment on alkaline phosphatase, osteonectin and type 1 collagen mRNA levels studied by northern analysis in hFOB cells in culture are shown in Fig. 3. Ethanol had no effect on steady-state mRNA levels for these markers of osteoblast differentiation and activity.

The dose (0-200 mM) effects of 7 days of ethanol treatment on alkaline phosphatase, type I collagen and osteonectin mRNA levels are shown in Fig. 4. Ethanol had no effect on the mRNA

levels for alkaline phosphatase. The type I collagen mRNA levels were decreased by 20% and 46% at 50 mM and 200 mM concentrations respectively, the latter change being statistically significant. Finally, ethanol had no effect on the mRNA levels for osteonectin at the 50 mM dose but decreased it by 31% at 200 mM concentrations, which was statistically significant.

The effects of ethanol on type I collagen protein levels after 24 hrs and 1 week ethanol treatment are shown in Fig. 5. The type I collagen protein levels did not change significantly after 24 hrs of ethanol treatment in hFOB cells in culture at both lower (50mM) and higher doses (200mM) whereas after one week it increased by 57% and 20% at 50mM and 200 mM concentrations of ethanol, respectively. The increase noticed at 50mM dose of ethanol after 1 week treatment was statistically significant (Fig. 5).

To determine whether the alcohol alters alkaline phosphatase activity, we measured the activity at two different doses of ethanol (50 and 200 mM) after 24 hrs and 1 week (Fig. 6.). The alkaline phosphatase activity was not significantly changed at either dose after 24 hrs. After 1 week treatment the alkaline phosphatase activity decreased and the changes were statistically significant at 50 and 200 mM doses of ethanol.

Effect of Ethanol on Cytokines and Growth factors

We studied the changes in steady state mRNA levels for selected cytokines that have been implicated in the regulation of bone formation and resorption. We analyzed the mRNA concentrations for members of interleukin (IL) family, interferons (IFN), tumor necrosis factor (TNF) and transforming growth factor-beta (TGFβ) by RNase protection assay (Table 1). While many cytokine genes were not detectable by our assay (Table 1), there were changes in the mRNA levels of TGF β 1 and TGF β 2 (Fig. 7). The levels of TGF β1 increased whereas the levels of TGF β 2 decreased with increase in ethanol concentrations (Figs. 7B and 7C). Linear regression analysis

revealed significant dose relationships for TGFβ1 (R = .81; P<.05) and TGFβ2 (R = -.50; P<.05). TGF β 3 mRNA was not detected by this assay both in control and ethanol treated cells.

DISCUSSION

Although alcohol abuse has been long associated with osteoporosis, uncovering the mechanism of ethanol-mediated bone loss has been a challenging task. Interpretation of human data is difficult because of the uncertainties that arise as a result of the many problems inherent to performing controlled experiments in alcoholics. Nevertheless, a consensus has arisen that alcohol inhibits bone formation in humans (2). Studies in laboratory animal models for alcohol abuse, which are much easier to control than human studies, have confirmed that ethanol inhibits bone growth, decreases bone formation and leads to osteopenia (11, 13, 20). However, these animal studies cannot distinguish a direct toxic effect of ethanol on osteoblasts from alternative indirect mechanisms, such as a detrimental response to more toxic metabolites, changes in circulating levels of hormones and other systemic factors altered and for local production of cytokines and other signaling molecules. The present dose response study using cultured normal hFOB (16) was designed to determine whether ethanol has a direct toxic effect on osteoblast growth and gene expression. This hFOB cell line has been well characterized and has been shown to respond to known regulators of osteoblast activity and gene expression (21-25). The results demonstrate that ethanol has mixed effects on indices of osteoblast differentiation and no significant effect on osteoblast number. These generally weak effects contrast to the robust response to alcohol observed in humans and laboratory animals at much lower concentrations of ethanol.

Blood alcohol levels of <20 mM are sufficient to rapidly reduce biochemical markers of bone formation in humans, as well as histological indices of bone formation in rats. Indeed, bone formation was significantly decreased in rats fed alcohol in their diet comprising as little as 3% of total caloric intake resulting in blood alcohol levels below 5 mM (R.T. Turner, unpublished data). On the other hand, our present study shows that a wide concentration range of ethanol (10-200 mM) had no short-term (1 day) effect on indices of osteoblast differentiation (alkaline phosphatase,

osteonectin and collagen mRNA levels). Additionally, alcohol had no detrimental effect on short or long term (7 days) accumulation of collagen in the media and, the alkaline phosphatase activity was only slightly reduced after long-duration of ethanol treatment. These results provide strong evidence that ethanol has minimal toxic effects on the mature osteoblast. We can not rule out the possibility that hFOB cells are more resistant to the direct toxic effects of alcohol than osteoblasts *in vivo*. If this were the case, however, it would represent a general *in vitro* phenomenon because other osteoblastic cell lines are similarly resistant to ethanol (26).

The lack of short-term effects of ethanol on expression of bone matrix protein genes in cultured osteoblasts contrasts with the dramatic *in vivo* response. Ethanol has transient tissue and dose dependent effects on steady-state mRNA levels for extracellular matrix proteins (collagen, osteocalcin and osteonectin) in rats (12). Acute ethanol treatment resulted in a coordinated increase in mRNA levels for all the matrix proteins in bone but not uterus and liver. However, continued treatment resulted in a decrease in mRNA levels for the matrix proteins as well as a decrease in bone formation (12). The results obtained in the present studies suggest that the changes in gene expression observed *in vivo* are not due to a direct effect of ethanol on the mature osteoblast. The relatively small inhibitory effects of very high concentrations of ethanol on mRNA levels for matrix proteins after prolonged exposure may have been mediated by the observed changes in TGFβ expression. The physiological significance of this proposed mechanism can be questioned because the changes in matrix protein gene expression were only observed at levels of ethanol unlikely to be observed in humans. Nevertheless, the apparent linear dose responses of TGFβ1 and TGFβ2 steady state mRNA levels suggest that expression of these cytokines might also be influenced by a lower concentration of ethanol. However, there is no *in vivo* data to support changes in TGFβ expression in bone.

With the exception of TGFβ1 and TGFβ2, we either could not detect or did not observe changes in gene expression of any of the other cytokines analyzed. TGFβ is an important signaling protein produced by osteoblasts that affects both bone formation and bone resorption (27). It promotes osteoclast apoptosis but inhibits osteoblast apoptosis thus exerting an influence on bone remodeling. Overexpression of TGFβ2 in transgenic mice deregulates bone remodeling leading to an age dependent loss of bone mass that resembles high-turnover osteoporosis in humans (28). These mice also have an increased rate of bone matrix formation which is not due to direct action of TGFβ but due to homeostatic response to the increase in bone resorption caused by TGFβ. These observations confirm that TGFβ is a physiological regulator of bone metabolism suggesting that it may have a role in mediating some of the effects of alcohol. The observed alterations in TGFβ by alcohol are not unique to osteoblasts. Ethanol mediated induction of TGFβ has been reported in macrophages (29). Also ethanol at physiologically relevant concentrations (25 mM) has been shown to induce TGFβ in monocytes (30).

The cells in our study were not exposed to uniform concentrations of ethanol; there was gradual decrease with time until the media was replaced which restored ethanol levels to the previous maximal concentrations. This pulsatile exposure, although imperfect, is likely to more accurately model the changes in blood levels in chronic drinkers than maintenance of constant levels of alcohol. The nearly identical time course changes in ethanol concentration in culture dishes with and without cells suggest that hFOB cells are unable to metabolize ethanol. This finding is significant because the metabolites of ethanol may be more toxic than the parent compound (31, 32).

A direct inhibition of osteoblast proliferation is supported by the work of Klein *et al.,* (26) who have shown that ethanol decreases [^{3}H]-thymidine incorporation and reduces cell growth in cultured TE85 osteosarcoma cells. Our observed tendency for a reduction in cell number is

generally consistent with the earlier result, but only at concentrations of ethanol unlikely to be observed in humans (200mM). Further studies will be necessary to determine the respective mechanisms of the cell specific ethanol-induced growth arrest in the two cell lines as well as the relevance for this response to the whole organism.

Finally, ethanol may have minimal effects on expression of osteoblasts in their basal state but may alter the response of these cells to external regulatory signals. Bone turnover is regulated by locally produced as well as systemic factors (27, 33-36). Disruption of one or more of these important signaling pathways could have detrimental effects on bone formation. This possibility is supported by Farley *et al.*, who have shown that ethanol disrupts the response of primary cultures on bone cells to several mitogenic agents (15). hFOB cells have not been shown to express mRNA for either IGF-I or TNF-α. This may be important to the failure to detect large effects of ethanol on these cells because those two cytokines are clearly altered in skeletal tissues *in vivo* (12). Whatever the specific mechanism by which alcohol indirectly inhibits bone formation, our finding that ethanol does not have a toxic effect on the osteoblast is important because it increases the likelihood that the detrimental effects of ethanol on osteoblast functions are reversible.

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Table 1. List of cytokines analyzed by RNase Protection assay in ethanol treated hFOB cells.

Interleukin-12 p35	Not detected
Interleukin-12 p40	Not detected
Interleukin-10	Not detected
Interleukin-6	Not detected
Interleukin-1 α	Not detected
Interleukin-1 β	Not detected
Interleukin-1 Ra	Not detected
Interferon- β	Not detected
Interferon- γ	Not detected
Lymphotoxin- β	Not detected
Tumor Necrosis Factor- α	Not detected
Tumor Necrosis Factor- β	Not detected
Transforming Growth Factor- β 1	Increased
Transforming Growth Factor- β 2	Decreased
Transforming Growth Factor- β 3	Not detected

Figure Legends

Fig. 1. Effect of 7 days treatment of ethanol on growth of hFOB cells. Cells were treated with 0, 50 and 200 mM concentrations of ethanol. The data represent the mean ± SE of three independent experiments. No significant changes were detected.

Fig. 2. Time course for ethanol present in the media. Culture dishes containing media in the presence and absence of hFOB cells were treated with 200 mM concentrations of ethanol. The data represent the mean ± SE of three independent experiments. *, $P \leq 0.05$ (compared to untreated control, by one way ANOVA and Fisher's PLSD analysis).

Fig. 3. Effect of ethanol at various doses after 1 day treatment on Alkaline phosphatase, Type 1 collagen and Osteonectin mRNA levels. Total RNA was isolated from cells treated with 0, 10, 50, 100 and 200 mM concentrations of ethanol and analyzed by northern blot hybridization using specific cDNA probes. The radioactive signal measured by PhosphorImager analysis has been expressed as a percentage of the value obtained from untreated controls. The data represent the mean ± SE of three independent experiments. No significant changes were detected.

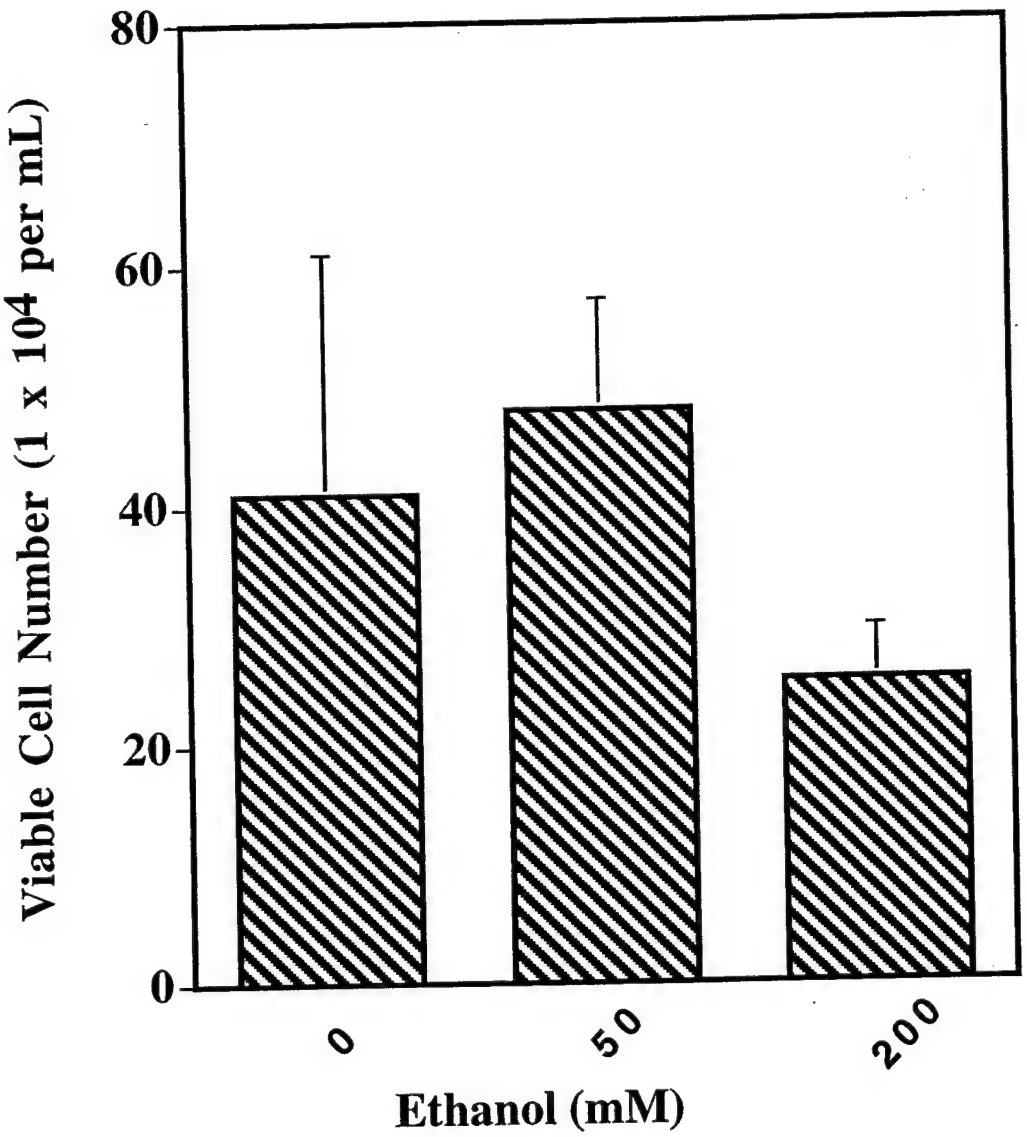
Fig. 4. Effect of ethanol at various doses after 1 week treatment on Alkaline phosphatase, Type 1 collagen and Osteonectin mRNA levels. Total RNA was isolated from cells treated with 0, 50 and 200 mM concentrations of ethanol and analyzed by northern blot hybridization using specific cDNA probes. The radioactive signal measured by PhosphorImager analysis has been expressed as a percentage of the value obtained from untreated controls. The data represent the mean ± SE of three

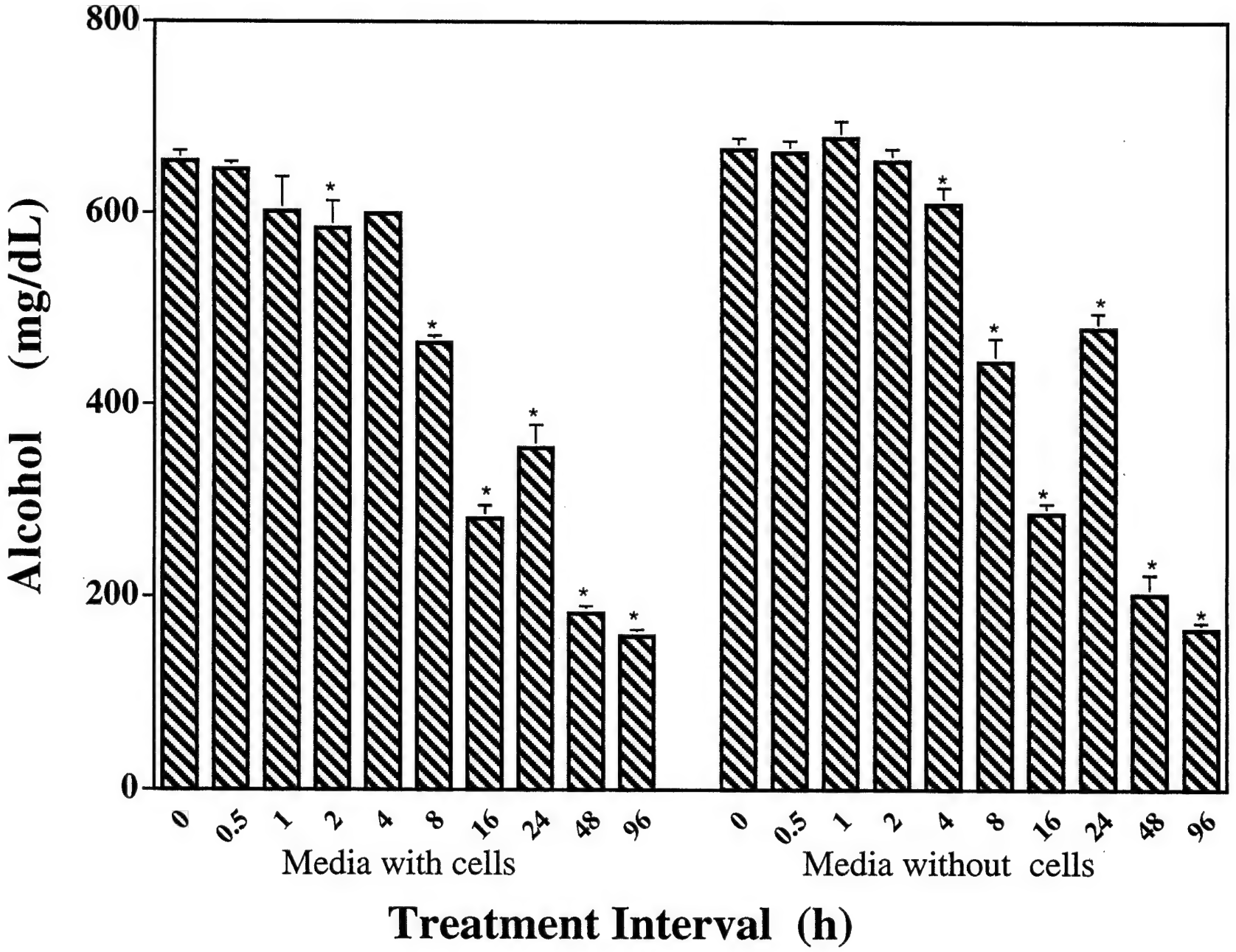
independent experiments. *, P ≤0.05 (compared to untreated control, by one way ANOVA and Fisher's PLSD analysis).

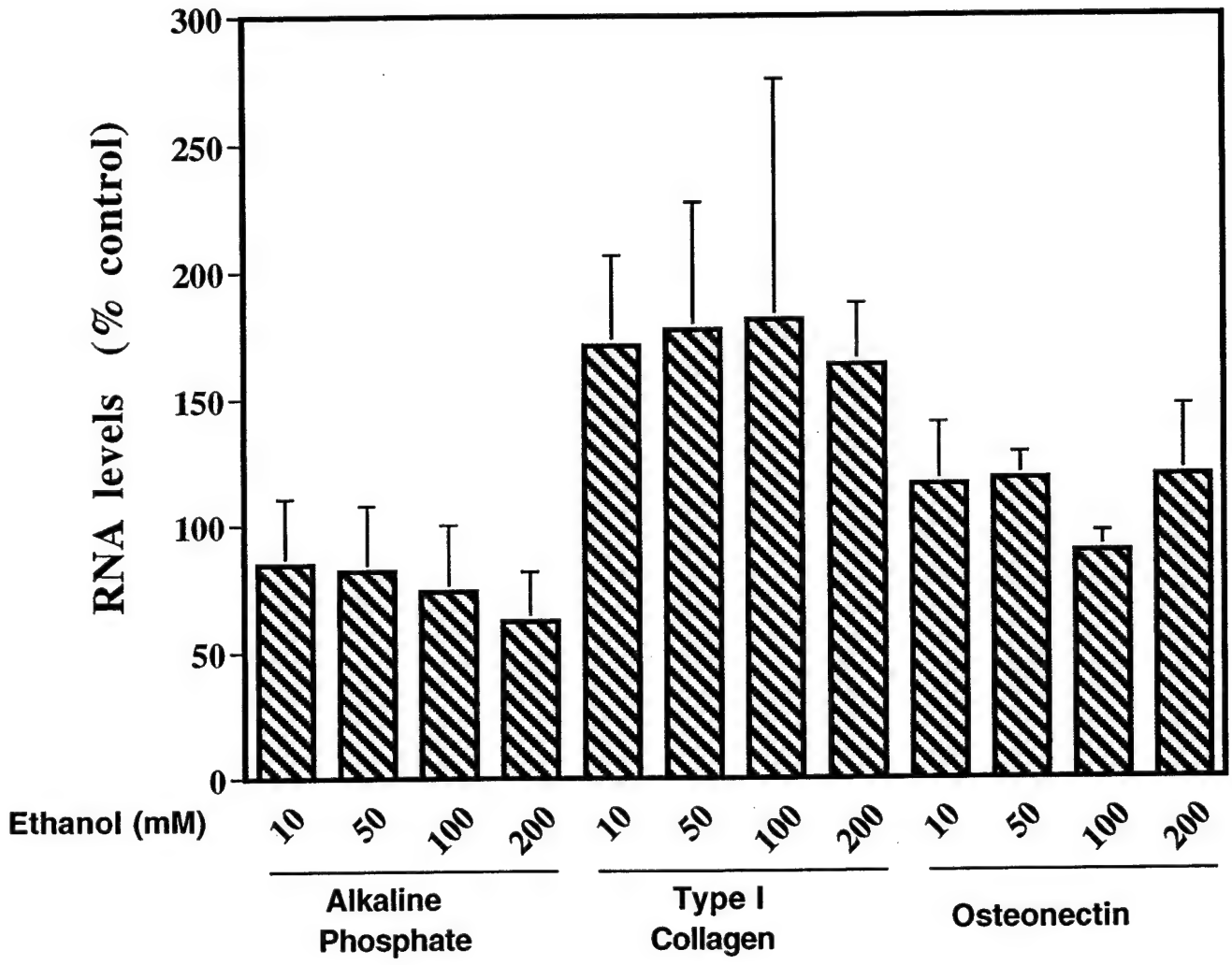
Fig. 5. Effects of 1 day (A) and 1 week (B) treatments of ethanol on Type 1 collagen protein levels in hFOB cells. Cells were treated with 0, 50 and 200 mM concentrations of ethanol and the Type 1 collagen levels in the medium was measured. The results represent the mean ± SE of three independent experiments. *, P ≤0.05 (compared to untreated control, by one way ANOVA and Fisher's PLSD analysis).

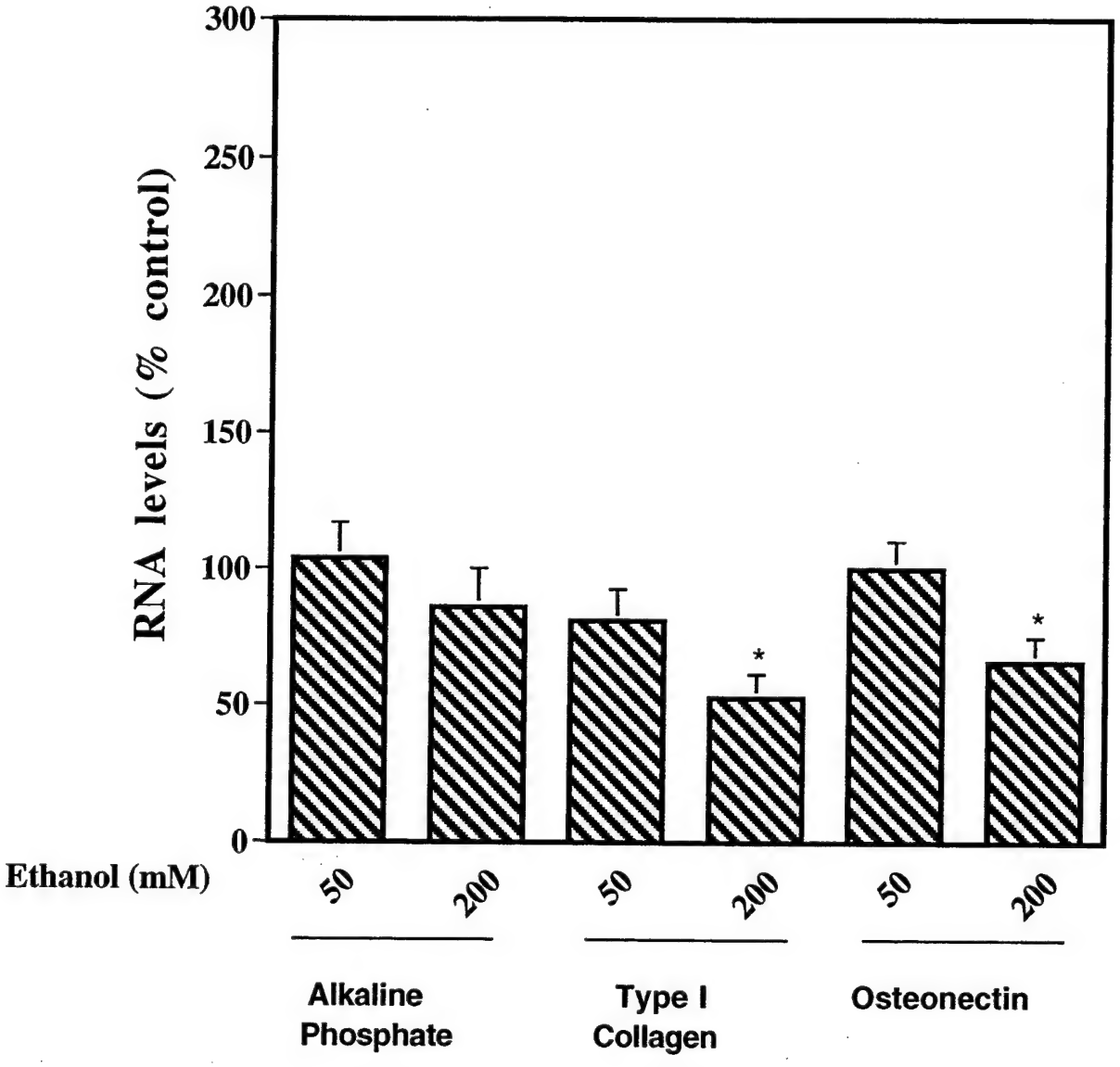
Fig. 6. Effects of ethanol treatment on Alkaline Phosphatase activity. The hFOB cells treated with 0, 50 and 200 mM concentrations of ethanol for 1 day and 1 week were harvested and the alkaline phosphatase activity in the cell pellet was measured. The results represent the mean ± SE of six replicate cultures from a single experiment. No significant changes were detected.

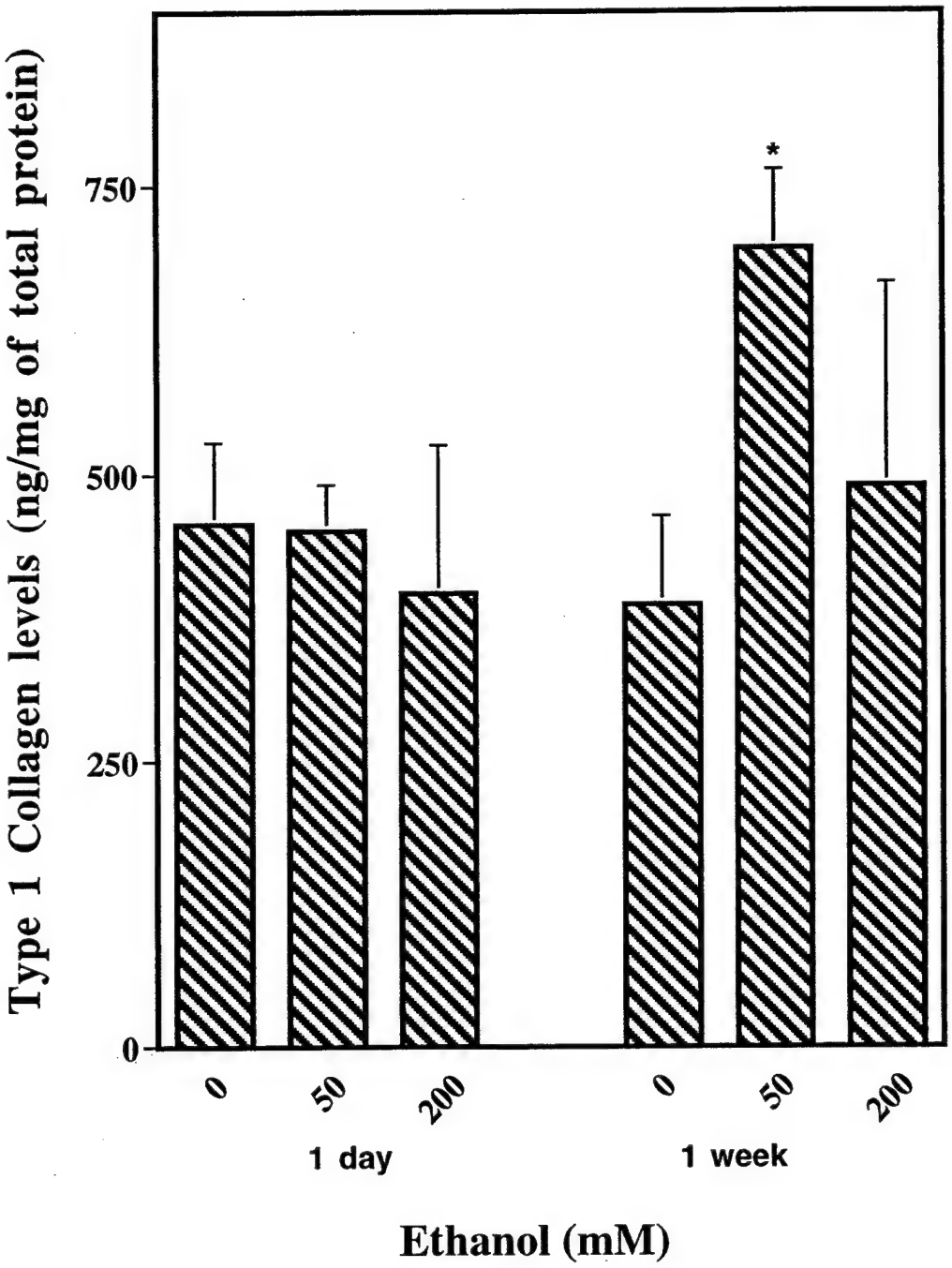
Fig. 7. Effect of 1-day ethanol treatment on TGF-β mRNA levels. Total RNA (10 μg) isolated from ethanol untreated and treated cells were analyzed by Ribonuclease Protection Assay. The radioactive signals from the gel (Fig. 7A) were measured by PhosphorImager analysis. The data points in diamonds (♦) are mRNA levels per ethanol dose for TGFß1 (Fig.7B) and TGFß2 (Fig.7C). The solid square (■) represents the mean for each dose. The mRNA response of TGFß1 and TGFß2 correlate significantly with ethanol doses (p≤0.002, p≤0.017, respectively).

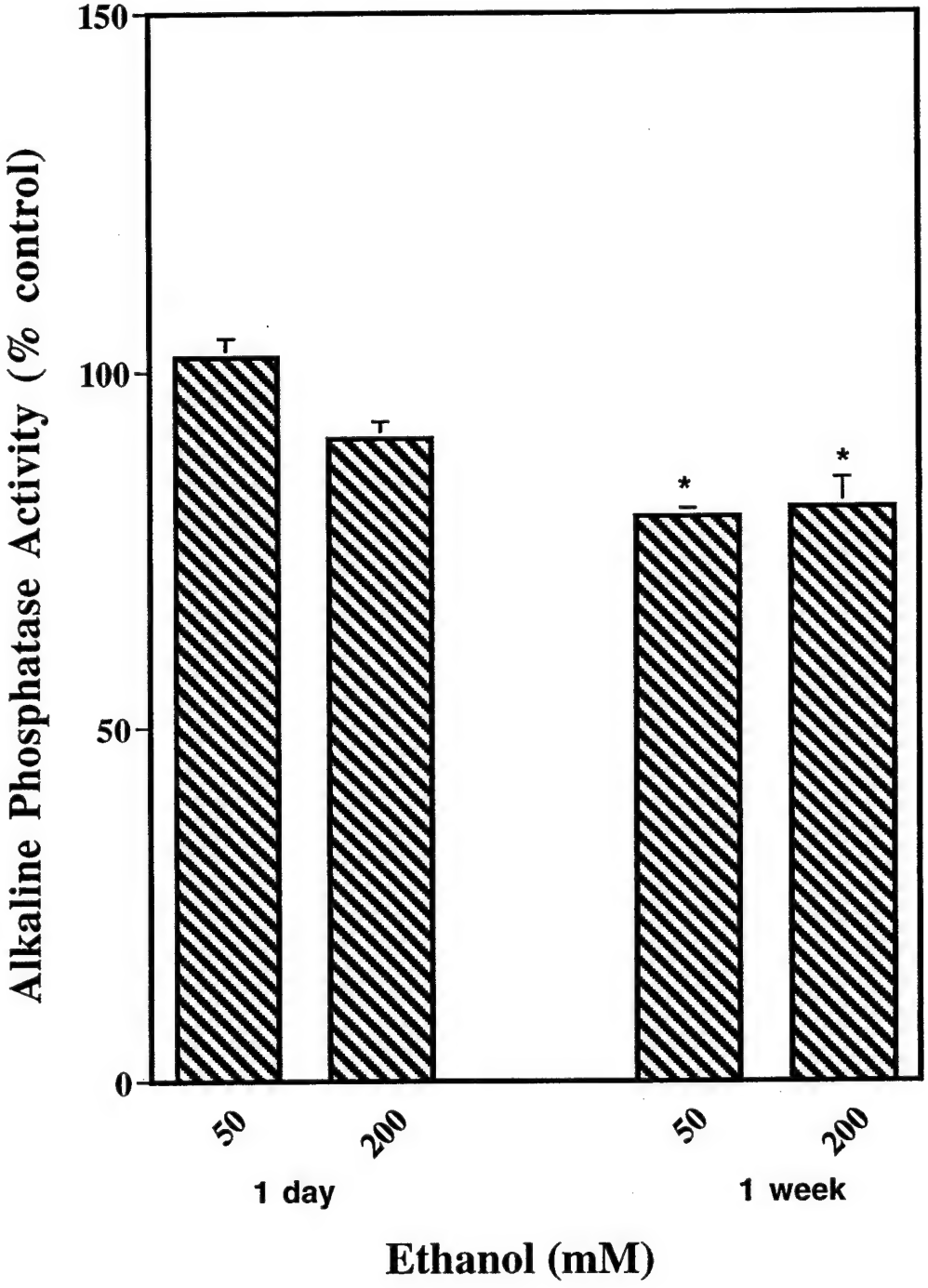

80
60
40
20
0
Viable Cell Number (1 x 10^4 per mL)
0
50
200
Ethanol (mM)

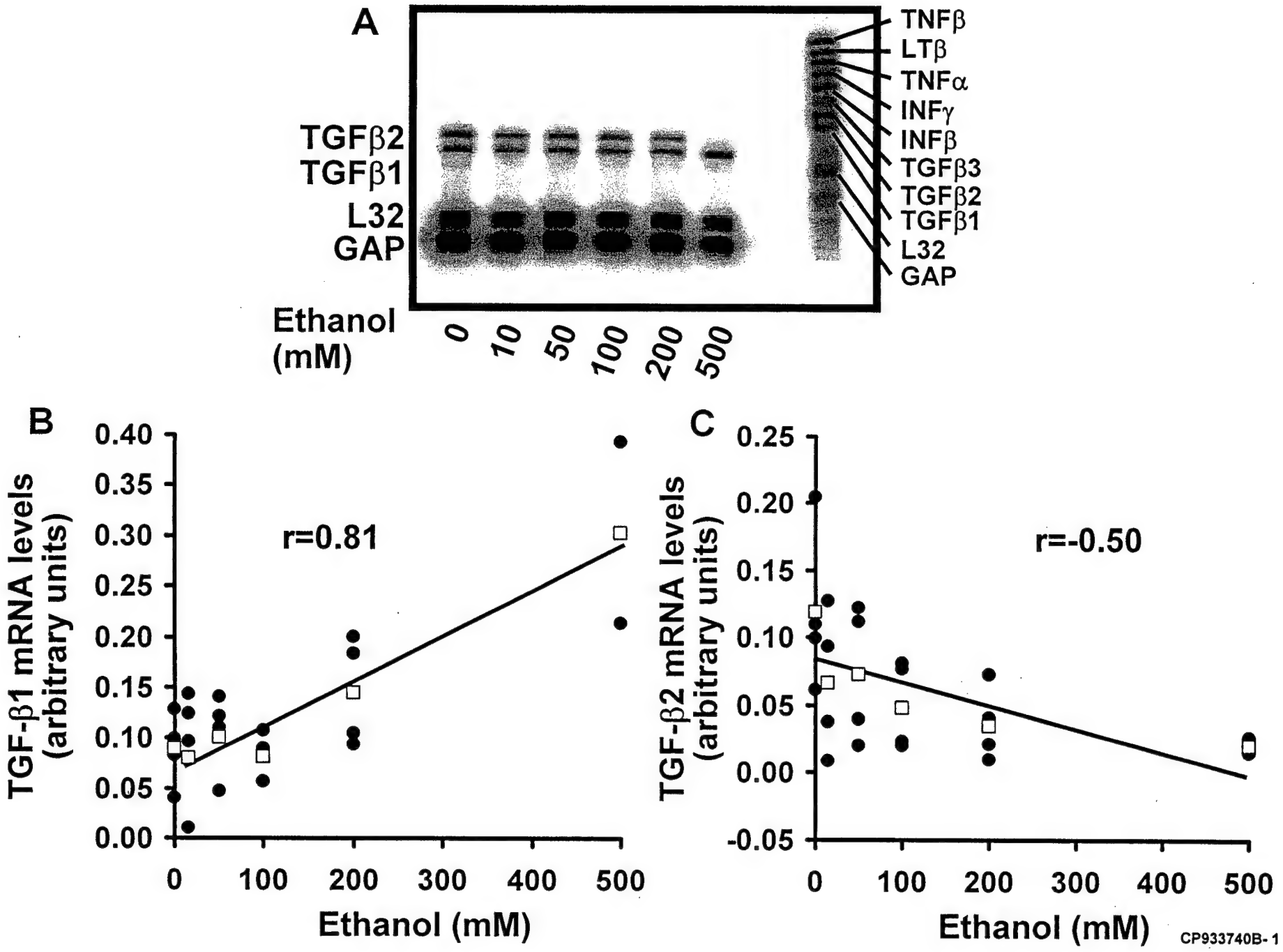

800
600
400
200
0
Alcohol (mg/dL)
0
0.5
1
2
4
8
16
24
48
96
Media with cells
Media without cells
Treatment Interval (h)


RNA levels (% control)
300
250
200
150
100
50
0
Ethanol (mM)
10
50
100
200
10
50
100
200
10
50
100
200
Alkaline Phosphate
Type I Collagen
Osteonectin


300
250
200
150
100
50
0
RNA levels (% control)
Ethanol (mM)
50
200
50
200
50
200
*
*
Alkaline Phosphate
Type I Collagen
Osteonectin


Type 1 Collagen levels (ng/mg of total protein)
750
500
250
0
*
0
50
200
0
50
200
1 day
1 week
Ethanol (mM)


150
100
50
0
Alkaline Phosphatase Activity (% control)
*
*
50
200
50
200
1 day
1 week
Ethanol (mM)


A
TGFβ2
TGFβ1
L32
GAP
Ethanol (mM)
0
10
50
100
200
500
TNFβ
LTβ
TNFα
INFγ
INFβ
TGFβ3
TGFβ2
TGFβ1
L32
GAP
B
TGF-β1 mRNA levels (arbitrary units)
r=0.81
Ethanol (mM)
C
TGF-β2 mRNA levels (arbitrary units)
r=-0.50
Ethanol (mM)
CP933740B-1

MODERATE ALCOHOL CONSUMPTION SUPPRESSES BONE TURNOVER IN ADULT FEMALE RATS

Running Title: Alcohol and Bone Turnover

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ABSTRACT

Chronic alcohol abuse is a major risk factor for osteoporosis but the effects of moderate drinking on bone metabolism are largely uninvestigated. Here we studied the long-term dose response (0,3,6,12, and 35% caloric intake) effects of alcohol on cancellous bone in the proximal tibia of eight-month-old female rats. After four months of treatment, all alcohol consuming groups of rats had decreased bone turnover. The inhibitory effects of alcohol on bone formation were dose dependent. A reduction in osteoclast number occurred at the lowest level of consumption but there were no further reductions with higher levels of consumption. An imbalance between bone formation and bone resorption at higher levels of consumption of alcohol resulted in trabecular thinning. Our observations in rats raise the concern that moderate consumption of alcoholic beverages in humans may reduce bone turnover and potentially have detrimental effects on the skeleton.

Key Words: rat bone, alcohol abuse, bone formation, bone resorption

INTRODUCTION

Alcoholics often have radiographic and histomorphometric evidence of osteopenia and a greatly reduced bone mineral density (1-3). Histomorphometric analysis of bone biopsies and measurement of biochemical markers of bone metabolism have consistently revealed evidence that alcohol excess inhibits bone formation (4-13). The effects of ethanol on bone resorption are less certain; increases, decreases and no change have been reported (2,4-7,11,13).

The inability to assign a role for bone resorption in mediating alcohol-induced bone loss highlights the difficulties associated with performing studies in alcoholics. Human studies are often difficult to interpret because of the small number of patients who can be studied and wide variations of the patient population in age, duration and pattern of alcohol abuse and accompanying risk factors. It is difficult to distinguish the direct effects of ethanol from secondary factors such as magnesium and zinc deficiency, reduced mechanical loading of the skeleton due to decreased physical activity and weight loss, malabsorption due to chronic pancreatitis, and skeletal abnormalities associated with increased cigarette smoking and increased use of aluminum-containing antacids. The role of abnormal liver function is especially controversial with some investigators reporting bone loss in alcoholics free of liver disease and others reporting no bone loss (4,8,12,14-17). It is especially difficult to control for nutrition, in part because alcoholics have a larger caloric intake than their peers but are frequently underweight (18,19).

A rat model for alcohol abuse has been developed to circumvent the limitations of human studies. Weight and nutrition can be carefully controlled in this animal model. Growing rats who consume ethanol at a rate (adjusted for the difference in body mass) comparable to alcoholics develop osteopenia and other abnormalities in bone and mineral metabolism (20,21).

All of the changes described in the rat model have been reported in alcoholic patients (2-4,6,7,11-13,15,17).

The skeletal response to low and moderate alcohol consumption are relevant to a much larger segment of the adult population than is alcohol abuse, but has not been extensively studied in either humans or laboratory animals. The present investigation was designed to investigate the long-term dose response effects of ethanol on the skeleton of adult female rats. Specifically, the study was designed to determine the minimum consumption of ethanol required to induce bone loss.

METHODS

Animal Experiment

Female Sprague Dawley rats (Harlan, Indianapolis, IN) were received at eight months of age (BW 279 ± 3 g, Mean ±SE). All subsequent procedures performed on animals were approved by the Mayo Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Rats were weight-matched into seven study groups (n=9-12) comprised of four ethanol-treated groups (3%, 6%, 13% and 35% caloric intake), a control group fed ad libitum, a control group pair-fed to the 6% ethanol-treated group and a control group sacrificed at the start of the experiment (baseline). As described for a previous study (22), rats were individually housed in a temperature- and humidity-controlled animal facility on a 12-h light/dark cycle. During the first week of the study, all animals were acclimated to a modified Lieber-DeCarli liquid diet (BioServe, Frenchtown, NJ). This diet contains 1.3 g/l calcium and 1.7 g/l phosphorous. Protein, fat and carbohydrates contribute 18, 35, and 47% of caloric intake, respectively. The alcohol-supplemented groups were subsequently fed, ad libitum, a liquid diet containing increasing concentrations of ethanol (95%

v/v) until receiving the appropriate percent of total caloric intake at the end of the first treatment week. Treatment continued for four months. Rats were not given water. Control rats did not receive ethanol and were fed a liquid diet isocalorically supplemented with maltose/dextran, as per the manufacturer's instructions. All animal weights were recorded weekly for the last 12 weeks of the study. On the first day of ethanol treatment, all rats were injected with a 20 mg/kg BW dose of a bone fluorochrome (tetracycline-hydrochloride; Sigma, St. Louis, MO) perivascularly at the base of the tail. The baseline control rats were sacrificed 24-h later. The remaining rats were injected (20 mg/kg BW) with the fluorochromes calcein (Sigma) and demeclocyline (Sigma) nine days and two days prior to sacrifice, respectively. Rats were anesthetized using ketamine and xylazine, weighed and sacrificed by decapitation. Tibiae were harvested, defleshed and fixed by immersion in 70% ethanol for bone histomorphometry. Wet weights of uterii were recorded.

Cancellous Bone Histomorphometry

Tibiae were processed for plastic embedding without demineralization and sectioned as described (22). Histomorphometry of cancellous bone was performed on unstained longitudinal sections (5 μm thick) in a standard sampling site in the proximal tibial metaphysis (22). Terminology and abbreviations are consistent with the standardized histomorphometric nomenclature (23).

All measurements were conducted on an image-analysis system employing OsteoMeasure software (OsteoMetrics, Atlanta, GA) as described (22).

The following measured and derived histomorphometric values were obtained: Total cancellous bone area measured at a magnification of x 10 in a metaphyseal sampling site divided by the total area of the tissue sampling site (2.88 mm^2) and expressed as a percentage.

Cancellous bone area was compared between the ad libitum and the pair-fed control groups to determine if there was a significant effect of diet on bone area. Trabecular thickness (Tb.Th), number (Tb.N), and separation (Tb.S) were estimated using the method of Parfitt, et al. (24).

Fluorochrome-based measurements and derived values consisting of double label perimeter, mineral apposition rate and bone formation rate were determined as described (22). The measurements were performed at a magnification of 10 x.

The length of cancellous bone perimeter covered by osteoclasts was measured in toluidine blue stained sections at 20 x, divided by total bone perimeter, and expressed as a percentage. Osteoclasts were morphologically distinguished as large, multinucleated cells with a foamy cytoplasm juxtaposed to bone surface.

Statistics

All values are expressed as means ± standard error. Significant differences between alcohol-treated groups and controls were determined by Fisher's Protected Least Significant Difference post hoc test for multiple group comparisons following detection of significance by 1-way ANOVA. Significance was considered at p values < 0.05. Dose response effects were evaluated by linear regression analysis.

RESULTS

Pair-feeding had no effect on any measured value. For this reason, the pair-fed and ad lib controls groups were combined.

The effects of ethanol on body weight, change in body weight, food and ethanol consumption and uterine weight are shown in Table 1. The initial body weights did not differ

between the treatment groups. Ethanol had minor effects on final body weight and food consumption. The lowest concentration of ethanol (3% caloric intake) increased final body weight, rate of change in body weight as well as consumption of the diet, whereas the highest concentration (35% of caloric intake) significantly decreased food consumption and rate of change in body weight. There was a near linear increase in total ethanol consumed/day as the concentration of ethanol was increased in the diet. Ethanol treatment tended to decrease uterine weight; the reductions were significant for the groups with 6% and 35% caloric intakes.

The effects of ethanol on static bone histomorphometry are summarized in Table 2. BA/TA was significantly decreased in the 13% and 35% caloric intake groups and Tb.Th was decreased for intake levels at and above 6%. Tb.N and Tb.S were not significantly influenced by ethanol.

The effects of ethanol on cancellous bone dynamic and cellular histomorphometry are summarized in Table 3. Ethanol treatment decreased mineralizing perimeter and bone formation rate (perimeter referent). Ethanol had no effect on MAR. Ethanol-treatment reduced osteoclast perimeter at all concentrations to a similar magnitude.

We were unable to measure longitudinal bone growth because of inadequate separation between the tetracycline label given at the start of the experiment and the mineralized hypertrophic cartilage.

The dose response effects of alcohol are summarized in Table 4. Linear regression revealed significant dose dependent decreases in BA/TA, Tb.Th, M.Pm and BFR. There was no dose dependent effect of alcohol on body weight, uterine wet weight, Tb.N, Tb.S, MAR, or Oc.Pm.

DISCUSSION

The observed results are disturbing because we did not observe a no-effect dose for alcohol consumption. Pronounced changes in bone metabolism were observed at the lowest consumption level of alcohol. Dietary intake of alcohol comprising as little as 3% of total calories dramatically reduced histological indices of bone turnover. Higher consumption levels of alcohol resulted in alterations in trabecular architecture and even net cancellous bone loss.

Analysis of the cancellous bone architecture revealed that alcohol-induced bone loss was due to a reduction in trabecular thickness; trabecular number was not altered. Osteoclast perimeter was not increased, indicating that cancellous bone loss was due to a disturbance in the bone remodeling balance rather than increased bone remodeling. Indeed, the present results indicate that alcohol consumption results in reduced bone remodeling as evidenced by the decreases in histomorphometric indices of bone resorption (osteoclast number) and bone formation (mineralized bone perimeter).

Cancellous bone remodeling occurs when focal resorption (remodeling unit) is initiated on a previously quiescent trabecular surface (25). A small amount of bone is resorbed and the resulting resorption lacuna is filled shortly thereafter as a result of new bone formation. There are two cellular mechanisms which could lead to trabecular thinning: 1) excessive erosion during the resorption phase; and 2) incomplete refilling of the erosion cavity during the formation phase. The maximum inhibition of osteoclast number was achieved at the lowest level of alcohol consumption, whereas mineralized bone perimeter showed a striking dose-dependent decrease. These findings suggest that incomplete filling of the erosion cavity during the

formation phase of the remodeling cycle is the more likely cellular mechanism for trabecular thinning. The mineral apposition rate was not altered, suggesting that alcohol inhibits onset of the bone formation phase of the remodeling cycle but not its continuation once initiated. These results are consistent with Dyer, et al. (26) who concluded that alcohol inhibits osteoblast proliferation and activity in the rat. This observation is also consistent with in vitro studies demonstrating that ethanol delays recruitment of osteoblasts but has little effect on bone matrix protein gene expression and peptide secretion by mature osteoblasts (27,28). Similarly, ethanol did not increase apoptosis in vitro (25), suggesting that osteoblast lifespan is unaffected.

At first glance, the observed non-dose dependent reduction in uterine weight in rats fed some doses of ethanol suggests that gonadal insufficiency contributes to the bone changes. However, the histological changes are not consistent with this possibility. Ovariectomy results in greatly elevated bone turnover (29,30) whereas alcohol reduced bone turnover. Also, the pattern of bone loss differs from ovariectomy. Whereas gonadal insufficiency decreases trabecular number, ethanol resulted in a decrease in trabecular thickness (22). The mechanism for the uterine atrophy is not clear but alcohol has been reported to induce a variety of pathophysiological changes in the uterus, including uterine atrophy (31). Furthermore, we have identified numerous genes whose expression are dramatically altered following acute administration of 1 mg/kg of ethanol, suggesting that alcohol has direct effects on the uterus (Turner et al., unpublished results).

Studies performed in growing male (21,32-35) and female (36,37) rats demonstrated that chronic consumption of large doses of alcohol in the diet suppresses bone growth. The resulting relative osteopenia is due to the failure to acquire a normal bone mass and is relevant to juvenile alcohol abusers. The present study in older rats is more relevant to adult humans and

demonstrates alcohol-induced bone loss in rats. Our failure to detect measurable longitudinal bone growth at the proximal tibial growth plate provides definitive evidence that the observed changes were not influenced by growth. The present study also differs from previous work in that it attempts to model moderate as well as abusive alcohol consumption.

There is no universal agreement as to what constitutes moderate drinking. Additionally, the level of ethanol consumption in rats in this study cannot be directly related to humans because the rates of metabolism differ between the two species. The variables that must be considered and evaluated in these studies include: total ethanol consumed, the % caloric intake contributed by ethanol and peak blood levels of ethanol.

The low dose group (3% caloric intake) consumed approximately 0.4 ml ethanol/day. On a body mass basis, this would be equivalent of ~3 standard drinks by a 50 kg woman, which is on the high end of moderate alcohol consumption. Relative to caloric intake, however, this level of consumption would be the equivalent of <0.5 daily drinks, which is on the low end. Blood ethanol levels may be more important than the absolute amount of alcohol consumed. Ethanol administered at 35% of caloric intake resulted in measured blood ethanol levels of 0.09-0.015% (21,32,33,36). The measured levels are likely to underestimate peak blood alcohol levels because sequential measurements have not been performed throughout the rat feeding cycle. Nevertheless, these levels are near to or exceed the impairment level which is generally considered to be between 0.08-0.10%. This high blood alcohol level contrasts with the 3% caloric intake dose rate, which results in blood alcohol levels below the assay detection limit. Taken as a whole, the data suggest that our dose range in the rat extends from the human equivalent of low-moderate to alcohol abuse.

Bone formation is generally reduced in alcoholics (3,4,7,17). Additionally, administration of ethanol to healthy volunteers results in an acute decrease in serum osteocalcin levels (8-10). The similarity between our results and those seen in humans indicate that in addition to being a good model for alcohol abuse in adults, the mature rat may also be predictive for the skeletal effects of moderate drinking.

The implication of a reduction in bone turnover in moderate drinkers may depend upon age and other factors. On one hand, ethanol consumption by adolescents might reduce peak bone mass, which would predispose the individual to osteoporosis. On the other hand, a reduction in bone turnover is likely to reduce the risk of osteoporosis in postmenopausal women because these individuals are losing bone at a rapid rate due in part to elevated bone turnover. This speculation is supported by epidemiological data indicating that postmenopausal moderate drinkers have a higher bone mass than abstainers (38-41). It is also supported by studies in ovariectomized rats which demonstrate that ethanol does not accelerate the bone loss associated with gonadal insufficiency and may reduce osteoclast number (22,42,43).

Neither moderate nor high consumption of alcohol prevented bone loss in ovariectomized rats (22,42,43). Ovariectomy is likely to result in a more extreme depletion of gonadal hormones than menopause. Epidemiological studies suggest that estrogen replacement accentuates the putative beneficial skeletal response to alcohol in postmenopausal women (41). Thus, it is possible that the combined anti-remodeling actions of estrogen replacement and alcohol have additive effects in women and rats.

In summary, these studies in rats demonstrate that alcohol consumption results in dose dependent bone loss and decreased bone turnover. A no-effect dose for alcohol was not observed. The findings in rats suggest that even moderate levels of alcohol beverage

consumption in humans may reduce bone turnover and potentially have detrimental effects on the skeleton.

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Table 1.—Body and Uterine Weights

Group	Initial BW (g)	Necropsy BW (g)	Change in BW (g/d)	Diet consumed (ml/d)	Ethanol consumed (ml/d)	Uterine Wt (g)
Control	277±5	321±9	0.40±0.06	67.8±2.3	0	0.931±0.047
3% Ethanol	279±6	358±15*	0.75±0.10*	76.6±1.5*	0.44±0.01*	0.946±0.069
6% Ethanol	281±5	339±12	0.54±0.12	69.3±1.4	0.80±0.02*	0.778±0.063*
13% Ethanol	272±9	323±15	0.47±0.08	64.1±1.3	1.60±0.03*	0.835±0.078
35% Ethanol	285±6	291±7	0.06±0.03*	61.5±2.4*	4.12±0.16*	0.722±0.045*

Values are mean ± SEM.

*P < 0.05 treatment (n=9-12 vs. control (n=20-22).

Body weight (BW)

Table 2.—Cancellous Bone Architecture

Group	BA/TA (%)	Tb.Th (μm)	Tb.N (mm^{-1})	Tb.S (μm)
Baseline	25.6±1.3	70.1±1.9	3.7±0.2	208±13
Control	20.6±1.2	64.9±2.6	3.2±0.1	260±15
3% Ethanol	19.5±1.4	62.5±3.0	3.1±0.1	267±16
6% Ethanol	18.3±1.7	57.6±2.3*	3.2±0.2	274±26
13% Ethanol	15.7±2.3*	52.5±2.4*	2.9±0.4	359±73
35% Ethanol	15.8±1.0*	50.9±1.7*	3.1±0.1	281±20

Values are mean ± SEM.

* $p < 0.05$ treatment (n=8-11) vs. control (n=19).

Bone area (BA)/tissue area (TA), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.S).

Table 3.—Fluorochrome and Osteoclast Measurements

Group	M.Pm/B.Pm (%)	MAR (μm/d)	BFR/BV (%/d)	Osteoclast Perimeter (%)
Control	11.05±0.75	0.94±0.04	0.325±0.019	15.77±1.15
3% Ethanol	5.79±1.07*	0.92±0.02	0.169±0.032*	10.78±1.85*
6% Ethanol	5.14±0.78*	0.91±0.03	0.171±0.031*	10.62±1.18*
13% Ethanol	3.71±0.77*	1.01±0.04	0.150±0.035*	10.66±0.99*
35% Ethanol	2.37±0.56*	0.86±0.04	0.077±0.019*	10.09±1.52*

Values are mean ± SEM.
*$p<0.05$ treatment (n=11) vs control (n=18-20).
Mineralizing perimeter (M.Pm), bone perimeter (B.Pm), mineral apposition rate (MAR), bone formation rate (BFR), osteoclast perimeter (Oc.P).

Table 4. -- Dose Response Effects of Ethanol Analyzed by Linear Regression

Measurement	r-value	p-value
Necropsy body weight (g)	--	NS
Uterine wet weight (g)	--	NS
BA/TA (%)	.31	.0001
Tb.Th (μm)	.43	.0012
Tb.N (mm^{-1})	--	NS
Tb.S (μm)	--	NS
M.Pm/B.Pm	.77	.0001
MAR (μm/d)	--	NS
BFR (%/d)	.79	.0001
Oc.Pm	--	NS

Bone area (BA)/tissue area (TA), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.S), mineralizing perimeter (M.Pm)/bone perimeter (B.Pm), mineral apposition rate (MAR), bone formation rate (BFR), osteoclast perimeter (Oc.Pm).

Skeletal Response to Alcohol

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ABSTRACT

This review briefly assesses the well-established effects of alcohol consumption on bone and mineral metabolism and addresses areas of controversy needing additional research to resolve. Alcohol consumption is a risk factor for osteoporosis based on the frequent finding of a low bone mass, decreased bone formation rate, and increased fracture incidence in alcoholics. Alcohol has also been shown to reduce bone formation in healthy humans and animals, and to decrease proliferation of cultured osteoblastic cells. On the other hand, it has been difficult to demonstrate alcohol-induced bone loss and increased fracture rate in population-based studies. Indeed, most population-based studies have shown a positive association between alcohol and bone mass and no change or a decrease in fracture risk. Overall, the evidence generally supports a detrimental effect of chronic alcohol abuse on the skeleton of men and a neutral or generally beneficial effect of light to moderate alcohol consumption, especially in older women. This latter putative beneficial effect may be due to a reduction in the age-related increase in bone remodeling associated with postmenopausal bone loss. Specific areas of research are recommended to clarify the dose and gender effects of alcohol consumption and to determine cellular and molecular mechanisms of action. The goals of this proposed research emphasis are to determine the degree of risk for the range of alcohol consumption, to set guidelines of consumption compatible with maintaining bone health, and to develop appropriate countermeasures to prevent or reverse the detrimental skeletal effects of alcohol abuse.

Introduction

This article will review the effects of alcohol on bone and mineral metabolism. It will emphasize the association between alcohol consumption and osteoporosis. Additional goals are to identify areas of controversy and suggest priorities for future research.

Effects of Alcohol Abuse on Bone Turnover in Humans

Histological studies suggest that alcohol abuse is associated with osteopenia due in part to decreased osteoblast activity (Bikle et al., 1993; Schnitzler and Solomon, 1984). The histological evidence for decreased bone formation is supported by consistent findings of reduced serum osteocalcin, a biochemical marker of bone formation (Gonzalez-Calvin et al., 1993; Labib et al., 1989; Laitinen et al., 1992; Laitinen et al., 1991; Laitinen et al., 1994; Nielsen et al., 1990; Rico et al., 1987). In contrast, the reported effects of alcohol abuse on histological and biochemical markers of bone resorption are contradictory, with evidence for no change, decreased and increased bone resorption reported (Bikle et al., 1985; Bikle et al., 1993; Crilly et al., 1988; Diez et al., 1994; Laitinen et al., 1991; Laitinen et al., 1994; Lalor et al., 1986; Schnitzler and Solomon, 1984).

There is evidence that duration of alcohol abuse is positively associated with the severity of osteopenia, suggesting that the bone loss is gradual (Harding et al., 1988; Odvina et al., 1995; Pumarino et al., 1996). A slow rate of bone loss suggests a small imbalance between bone formation and bone resorption that allows bone resorption to predominate as the principal cellular mechanism leading to osteopenia. The conclusion of some investigators (Gonzalez-Calvin et al., 1993; Laitinen et al., 1991; Laitinen et al., 1994) that bone remodeling is uncoupled

(bone formation is decreased and bone resorption increased) is difficult to reconcile with an apparent gradual rate of bone loss. When it occurs in other medical conditions, including immobilization and glucocorticoid excess, uncoupled bone remodeling results in significant bone loss within months (Leblanc et al., 1990; Reid, 1999). However, there have been no longitudinal studies so it cannot be excluded that alcohol-induced bone loss is punctuated, with brief periods of rapid bone loss followed by prolonged intervals with little or no change in bone mass.

The Effects of Moderate Drinking on the Human Skeleton

Long-term detrimental effects of alcoholism to reduce bone mass, although not universal, are relatively well established. In contrast, the skeletal effects of moderate alcohol consumption on bone and mineral homeostasis are relevant to a greater number of people but are less certain. Reports of improved bone mass, especially among postmenopausal women, are intriguing but not fully understood (Feskanich et al., 1999; Laitinen et al., 1993; Laitinen et al., 1991; Orwoll et al., 1996).

Mechanisms of Action of Alcohol on Bone Metabolism

The mechanisms of action of alcohol on bone turnover are not understood and may include both direct and indirect actions. The indirect actions may occur secondarily to changes in calcium regulating hormones, mineral homeostasis and mechanical loading (due to decreased body weight). Alcoholics often become hypomagnesmic, hypocalcemic, and may have hypocalciuria (Bikle et al., 1985; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Laitinen et al., 1992; Lalor et al., 1986). Vitamin D metabolism can also be disturbed. In patients with cirrhosis, intestinal absorption of vitamin D is diminished and the half-life of the labeled vitamin after intravenous administration is shortened (Barragry et al., 1979). Serum 25-hydroxyvitamin D [25(OH)D] was normal or reduced in patients with alcoholic liver disease (Hepner et al.,

1976). Serum levels of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] were normal or reduced in alcoholics, and serum parathyroid hormone levels were normal or elevated (Bikle et al., 1985; Bouillon et al., 1984). 1,25$(OH)_2$D can influence bone metabolism directly or via its actions to stimulate secretion of parathyroid hormone. Thus, it is possible that some of the effects of chronic alcohol abuse on bone mass are mediated by disturbances in the regulation of these important calcium-regulating hormones. However, no cause and effect relationship has been established and abnormal calcium homeostasis is not a universal finding in alcoholics. Again, most studies have emphasized the effects of alcohol on mineral homeostasis in alcoholics; the possible effects of alcohol on light to moderate drinkers are even less clear, and may differ both qualitatively and quantitatively from the effects of alcohol abuse. Another complication is that underage drinking may reduce peak skeletal mass creating a lower threshold for development of osteopenia later in life. However, there have been no human studies focusing on the effects of adolescent drinking on bone and mineral homeostasis.

The evidence for direct effects of alcohol on bone cell metabolism was obtained from cell culture studies. Alcohol was reported to increase indices of bone resorption in isolated osteoclasts and decrease indices of osteoblast differentiation (e.g., alkaline phosphatase activity) in osteoblast-like cells (Chavassieux et al., 1993; Cheung et al., 1995). High concentrations of alcohol inhibit the proliferation of osteosarcoma cells but do not appear to reduce cell lifespan (Klein and Carlos, 1995; Klein et al., 1996). This latter finding suggests that alcohol does not have a direct toxic effect on mature osteoblasts. It is especially interesting that alcohol interacts with skeletal signaling peptides to modify the response of bone cells to these important regulatory peptides (Farley et al., 1985). Although extrapolation of cell culture data to physiological systems must be viewed with extreme caution, these observations support the

hypothesis that osteoblast activity and cell signaling are disturbed by alcohol. Unfortunately, in vitro studies are unable to address the effects of alcohol on recruitment of and initiation of bone remodeling by osteoclasts and there are no established in vitro models for studying the coupling of bone formation to bone resorption.

Regulation of Bone Balance in Humans and Laboratory Animals

Bone is remodeled continuously throughout life by a process that was described by Frost (Frost, 1963). There is indirect evidence that bone remodeling, which is essential to maintain normal bone mass and architecture in the mature skeleton, can be disturbed by alcohol. Figure 1, which was adapted from Parfitt, illustrates the bone remodeling cycle (Parfitt, 1984). Cancellous bone remodeling occurs when a foci of resorption is initiated on a previously quiescent bone surface. A discreet unit of bone is removed and the resulting resorption lacuna is filled with new bone shortly thereafter. The formation phase of the bone remodeling sequence is mediated by osteoblasts who replace the osteoclasts in the resorption lacuna and can theoretically underfill, precisely fill or overfill the lacuna, resulting in a small decrease, no change, or small increase in bone volume, respectively.

The overall rate of bone remodeling is determined by the number of remodeling units. During the bone remodeling sequence, bone formation follows bone resorption and as a consequence is coupled to bone resorption. Because of coupling, changes in the rate of bone resorption result in delayed corresponding increases or decreases in bone formation. Changes in bone mass (ΔM) for a given time interval are determined by the relationship $\Delta M = \Sigma_{1\rightarrow N} (R\text{-}F)_1 + (R\text{-}F)_2 + (R\text{-}F)_3 \ldots\ldots\ldots (R\text{-}F)_N$ where "N" is the number of remodeling units, "F" is bone formation, "R" is resorption, and "R-F" is the net difference between formation and resorption for a completed or partially completed remodeling unit. Thus, increasing the rate of bone

remodeling will inevitably result in bone loss (because resorption precedes formation) which will be accentuated if bone formation does not completely refill the resorption cavity (R-F < 0).

Peak blood alcohol levels and duration of exposure to alcohol are two variables that are likely to influence the skeletal response to ethanol (Turner et al., 1998). However, there have been no experimental studies in human subjects and few in laboratory animals that have focused on dose dependent changes in bone remodeling. Epidemiological studies suggest that infrequent to moderate alcohol consumption decreases the bone remodeling rate (N) without disturbing remodeling balance (R-F) (scenario B in Figure 1), whereas alcohol abuse decreases N and creates a negative remodeling balance (scenario A in Figure 1). The exact dose response for either N or R-F remains to be determined and is likely to be influenced by additional factors such as age, sex and gonadal status.

The apparent contradictory effects of alcohol on bone mass in young men and postmenopausal women can be reconciled by considering the effects of drinking on bone remodeling. An imbalance in bone formation and resorption in young males in which the latter predominates would lead to gradual bone loss in spite of decreased bone remodeling. In contrast, an alcohol-induced decrease in bone remodeling in older women would slow bone loss relative to their peers in spite of the remodeling imbalance. In women, gonadal insufficiency following menopause results in greatly accelerated bone remodeling with a net increase in bone resorption (Heaney et al., 1978). Inhibitors of bone remodeling (such as estrogen) reduce the rate of bone loss (Felson et al., 1993). Thus, an inhibitory effect of moderate alcohol intake on initiation of the bone remodeling cycle would be consistent with studies reporting a relative (compared to age-matched women who are losing bone) improvement in bone mass in postmenopausal women.

Another type of bone turnover allows bone formation and bone resorption to be independently changed in magnitude and can lead to architectural changes. This process is called modeling and it need not involve a coupled response between bone formation and resorption (Turner, 1994). The relative contributions of altered bone modeling and remodeling to the bone loss induced by chronic alcohol abuse are not known.

As eluded to, the relationship between bone resorption by osteoclasts and bone formation by osteoblasts is central to achieve and maintain a neutral bone balance. This is analogous to a biochemical pathway where positive (activator) and negative (inhibitor) factors play a critical role in regulating the balance between opposing processes in the system. Local signaling peptides (growth factors and cytokines) appear to act as the biochemical messengers between the two classes of cells (Wergedale et al., 1986). In order for osteoclasts to resorb bone, first they have to be formed and then they have to become activated by contact with resorbable bone matrix (Vaes, 1988). The latter process requires removing both an osteoblast cell layer and an underlying layer of nonmineralized matrix (or osteoid tissue) (Vaes, 1988). It has been proposed that osteoblast lineage cells might regulate bone resorption in part by secreting enzymes to both remove the osteoid layer and activate osteoclasts (McSheehy and Chambers, 1986; Vaes, 1988). The osteoclast, however, is responsible for resorbing the organic as well as mineral phase of bone.

There is evidence for communication from osteoclasts to osteoblasts to create tighter interaction between the two metabolic processes of formation and resorption (McSheehy and Chambers, 1986; Vaes, 1988). The factors involved in this communication have been termed "coupling factor(s)" (Linkhart et al., 1986). An osteoclast-derived factor, which affects osteoblasts, has not been identified. However, non-osteoclast-derived peptides have been

implicated as coupling factors (Howard et al., 1981; Linkhart et al., 1986; Pfeilschifter et al., 1988). These peptides are produced by osteoblasts, trapped in bone matrix during bone formation, and quantitatively released by the resorbing activities of osteoclasts (Turner et al., 1988). Such a mechanism is hypothesized to promote osteoblast activity to follow bone resorption. Recent studies have demonstrated that the final step in osteoclast differentiation is regulated by osteoblasts and certain marrow cells (Simonet et al., 1997). Alcohol may alter the rate of initiation of bone remodeling as well as the coupling between bone formation and bone resorption by disturbing the local expression of cytokines which mediate these processes. In support of this hypothesis, the expression of two important cytokines, IGF-I and TNF-α, have been shown to be altered in bone by alcohol (Turner et al., 1998).

Limitations of Human Studies

Human studies in alcoholics are often difficult to interpret. Most have been underpowered because of the relatively small number of patients studied and wide variations in age, duration and patterns of alcohol abuse and other risk factors. The precise effects of alcohol on the human skeleton are not known because it is difficult to distinguish the specific effects of ethanol from co-morbidity factors such as poor nutritional status, magnesium and zinc deficiency, reduced mechanical loading due to decreased exercise and weight loss, malabsorption related to chronic pancreatitis, cigarette smoking, and use of aluminum-containing antacids. It is difficult to get an accurate assessment of lifelong alcohol consumption and inaccurate reporting can compromise interpretation of the results. The role of abnormal liver function is especially controversial, with investigators reporting bone loss (Bikle et al., 1985; Laitinen et al., 1992) as well as no bone loss (Crilly et al., 1988; Harding et al., 1988) in

alcoholics free of liver damage. One possible explanation for the variable results is that the age, weight, and nutritional status of the individual may be as important as the duration and magnitude of exposure to alcohol with older (Bikle et al., 1993) and lighter weight (Crilly and Delaquerriere-Richardson, 1990) abusers being more subject to bone loss than younger and heavier individuals.

Laboratory Animal Models

Studies of the effects of ethanol on bone and mineral homeostasis suggest that laboratory animals can provide additional insight into the specific effects of alcohol. Alcohol given acutely results in transient hypocalcemia in rats and dogs (Peng et al., 1972; Peng and Gitelman, 1974). Alcohol given chronically to growing male rats causes decreases in bone strength, density and cancellous bone volume (Peng et al., 1988; Turner, Aloia et al., 1988; Turner et al., 1991; Wezeman et al., 1999). Alcohol also suppresses overall growth. When pair feeding was used to insure comparable growth between the controls and alcohol-treated rats, consumption of alcohol comprising 36% of caloric intake for three weeks clearly inhibited bone formation at the tibial diaphysis, resulting in a delay in bone mineralization, and decreased bone strength (Turner et al., 1987; Turner et al., 1991). Alcohol consumption did not alter serum concentrations of either $24,25(OH)_2D$ or $1,25(OH)_2D$. Consumption of alcohol for 10 months resulted in cortical bone loss due to decreased bone formation (Turner, Aloia et al., 1988). Chronic alcohol consumption also resulted in cancellous osteopenia, but the cellular mechanism for the net bone loss was not established with certainty. Following this long-term exposure to alcohol, serum 25(OH)D was increased but $1,25(OH)_2D$ was decreased. These results demonstrate that the overall changes in bone and mineral homeostasis associated with long-term alcohol abuse in humans also occur in laboratory animal models.

Similarly, alcohol inhibits bone growth and results in a reduced peak bone mass in female rats (Dyer et al., 1998; Hogan et al., 1997; Sampson 1998; Sampson et al., 1997; Sampson et al., 1998; Sampson et al., 1996; Sampson and Spears, 1999). Cessation of alcohol consumption did not result in sufficient catch-up growth to restore normal bone mass or mechanical properties (Sampson and Spears, 1999). As in growing males, alcohol induces osteopenia in female rats primarily by inhibiting bone formation (Dyer et al., 1998; Hogan et al., 1997; Sampson, 1998; Sampson et al., 1997; Sampson et al., 1998; Sampson et al., 1996; Sampson and Spears, 1999).

Alcohol did not inhibit bone formation or result in additional bone loss in growing ovariectomized rats (Kidder and Turner, 1998). Although one study reported a decrease in osteoclast number (Fanti et al., 1997), moderate to high consumption of alcohol did not prevent osteopenia induced by ovariectomy (Fanti et al., 1997; Kidder and Turner, 1998; Sampson and Shipley, 1997).

In most long-term studies, alcohol has been administered to rats in their diet. One study which sought to model binge drinking in rapidly growing rats by administering alcohol by gavage reported an increase in bone mass (Sampson et al., 1999). This unexpected finding suggests that additional factors such as age, gonadal status or the pattern of consumption may be important in determining the overall skeletal effects of alcohol.

There are no published studies reporting the long-term effects of alcohol on the skeleton of adult animals. However, a 4-month dose response study in which ethanol was fed to female rats who were 8 months old at the start of treatment has been performed. Alcohol treatment resulted in a dose-dependent decrease in cancellous bone volume compared to baseline values or controls (Figure 2). These findings demonstrate that chronic heavy alcohol consumption results in bone loss in the rat model. Especially disturbing was the finding that alcohol comprising as

little as 3% of the rats caloric intake significantly reduced bone turnover (Turner, unpublished data).

Clinical Significance of Musculoskeletal Disorders

The clinical importance of musculoskeletal disorders in the general population is greatly under appreciated. Musculoskeletal complaints represent the number 1 and 2 reasons why patients see physicians and are hospitalized, respectively (Praemer et al., 1992). The annual direct and indirect costs of musculoskeletal disorders in the United States exceed $125 billion. Direct treatment accounts for less than half of the economic burden; morbidity, mortality, and the value of lost productivity account for the remaining costs.

There are over 6 million bone fractures in the United States per year of which roughly 5% do not heal properly and require additional and sometimes costly interventions. The consequences of fractures are especially severe in the elderly. Hip fractures are arguably the most devastating common fracture with more than 275,000 occurring annually. Patients with hip fractures have a 20% 6-month mortality rate. Furthermore, an additional 20% will require long term institutionalization (>1 year) and a similar percentage will face loss of mobility (i.e., dependence on wheelchair or walker) (Cummings et al., 1985).

The aging of the American population will lead to future increases in the incidence of osteoporotic fractures. Osteoporosis is usually caused by a chronic imbalance in the bone remodeling cycle where bone resorption is not adequately compensated for by subsequent bone formation. The onset of bone loss typically precedes the increased risk of fractures by one or two decades (Cummings et al., 1985; Praemer et al., 1992) and is asymptomatic during this interval. Insidious bone loss combined with the great difficulty of restoring bone to an

osteopenic skeleton make it imperative to identify preventable risk factors for bone loss and effectively intervene prior to clinical manifestations.

Effects of Alcohol Abuse on Bone Mass and Fracture Incidence

Until recently, the development of osteoporosis is men was generally perceived as an insignificant problem and was often not diagnosed, but male osteoporosis represents approximately one-third of the cases. The increasing longevity is likely to lead to future increases in the incidence of osteoporotic fractures in males as well as females. The development of osteoporosis in men is often associated with alcoholism. In one study, 45 of 96 young to middle-aged male alcoholics showed radiographic evidence of osteopenia (Spencer et al., 1986). Other studies have reported decreases in bone mass in male alcoholics by densitometry and histomorphometry of iliac crest bone biopsies (Bikle et al., 1985; Bikle et al., 1993; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Pumarino et al., 1996; Schnitzler and Solomon, 1984). Histological studies suggest that alcohol abuse is associated with reductions in cancellous bone volume and thickness of individual trabeculae. However, not all studies have detected significant differences in bone mass between male alcoholics and non-alcoholics (Odvina et al., 1995; Pumarino et al., 1996).

Bone loss resulting in atraumatic fractures in women is most commonly associated with gonadal hormone deficiency (3). There is little evidence for bone loss in female alcohol abusers (Laitinen et al., 1993). However, the great majority of studies performed in alcoholics to assess the effects of alcohol abuse on bone and mineral homeostasis have been performed in men. As a result, this risk has not been established with certainty for women. Animal studies suggest that younger men and women who abuse alcohol share a similar risk but that postmenopausal women may be at a lower risk than elderly men.

Prospective, as well as retrospective, studies have been performed to establish an association between alcohol and bone mass. In contrast to studies in alcoholics, these population-based studies have generally shown no difference or a higher bone mineral density associated with alcohol consumption (Felson et al., 1995; Feskanich et al., 1999; Grainge et al., 1998; Hoidrup et al., 1999; Holbrook and Barrett-Connor, 1993; Jouanny et al., 1995; Krogsgaard et al., 1995; Laitinen et al., 1992; May et al., 1995; Orwoll et al., 1996; Perry et al., 1999). This apparently beneficial effect of alcohol is most notable in women (Felson et al., 1995; Feskanich et al., 1999; Hoidrup et al., 1999; Holbrook et al., 1993; Orwoll et al., 1996).

The effect of chronic alcohol abuse on fracture risk has not been extensively studied. Fractures were reported to be approximately four times as common in a series of 107 chronic alcoholics as in age-matched random controls (Kristensson et al., 1980). In another study, excessive alcohol consumption was identified in 7 men out of a series of 47 men sequentially referred to a metabolic bone center because of atraumatic fractures or radiographic osteopenia (Kelepouris et al., 1995). In a larger cross-sectional study, alcohol abuse was also identified as a significant risk factor for fractures in men (Kanis et al., 1999) and fractures were much more common in patients with alcoholic liver disease than with various forms of non-alcoholic liver disease (Lindsell et al., 1982). In other studies, alcohol consumption was found to be not associated with fractures (Mussolino et al., 1998; Naves Diaz et al., 1997), associated with decreased fracture risk (Nguyen et al., 1996), or the association was found to be to be gender dependent (Hoidrup et al., 1999. In the study by Hoidrup et al., alcohol abuse was associated with an increased fracture rate in men but not in women.

There are numerous factors that could contribute to the differences in fracture rate that have been reported, including the presence or absence of alcoholic liver disease and differences

between the skeletal sites measured (e.g., ribs and vertebrae). One of the more important factors may be trauma (Johnson et al., 1984). Peris et al., (1995) found that most fractures in alcoholics were associated with trauma rather than osteopenia.

There have been few studies on the effects of alcohol on fracture repair. The limited available data suggest that alcoholism does not lead to an increase in the incidence of non-unions, osteonecrosis or other complications (Nyquist et al., 1997; Nyquist et al., 1998), but is associated with an increase in healing time for transverse fractures (Nyquist et al., 1997).

Summary and Recommendations

Alcohol abuse should be considered a risk factor for osteoporosis based on the frequent finding of a low bone mass, decreased bone formation, and increased fracture incidence in alcoholics. Alcohol has also been shown to reduce bone formation in healthy humans and animals, and decrease proliferation of cultured osteoblastic cells. On the other hand, not all alcoholics exhibit a low bone mass. Furthermore, it has been difficult to demonstrate either alcohol-induced bone loss or increased fracture rate in population-based studies. Indeed, most have shown a positive association between alcohol and bone mass and no change or a decrease in fracture risk. Overall, the evidence generally supports a detrimental effect of chronic alcohol abuse on the skeleton of a sub-population of men and a neutral or generally beneficial effect for moderate alcohol consumption, especially in women. This latter putative beneficial effect may be due to a reduction in the increase in bone remodeling which in part mediates age-related bone loss.

The following areas need additional research to resolve controversies, establish degree of risk or to develop countermeasures to prevent or reverse the detrimental skeletal effects of alcohol abuse.

Fracture Rate

Many alcoholics are osteopenic and a low bone mass is an established risk factor for atraumatic fractures. Although there is no clear cut association between drinking and bone mass in the general population, the magnitude of risk can only be determined if the fracture incidence for alcoholics is known. Ideally, these data will distinguish between traumatic and atraumatic fractures in order to determine if osteopenia rather than an increased accident rate is the major contributing factor leading to fracture in alcoholics (Blake et al., 1997; Nordqvist and Petersson, 1996).

Bone Turnover

Alcohol inhibits bone formation in humans and growing animals but there are important controversies regarding its effects on bone turnover. Is alcohol toxic to osteoblasts in vivo? Does alcohol antagonize osteoblast differentiation? Does alcohol act directly on the osteoblast to suppress bone matrix synthesis and if there is direct inhibition, is it reversible? To what extent is the reduction in bone formation an indirect consequence of an overall reduction in bone remodeling? Does alcohol uncouple bone formation from bone resorption during bone remodeling? Do biochemical markers in blood and urine accurately reflect bone formation and bone resorption in alcoholics? There are existing data in the literature which support and refute each of these possibilities. The answers to these questions are relevant to gaining insight into the etiology of alcohol-induced osteoporosis and are important to the rational development of countermeasures. Future studies should not be limited to alcohol abuse nor should they be limited to one gender or one age. Studies emphasizing moderate drinking should be encouraged because of the huge potential impact on public health.

Mechanisms

The biochemical and molecular mechanisms of action of alcohol on bone cells are poorly understood. Animal and cell culture models are now available to advance our understanding of mechanisms and such studies could lead to novel interventions and should be supported.

Countermeasures

Studies investigating countermeasures to the detrimental effects of alcohol on bone and mineral metabolism are rare. Dramatic recent advances have been made toward prevention and reversal of postmenopausal osteoporosis using pharmacologic approaches. The possible application to alcoholics of approved drugs which inhibit bone resorption and drugs under development which stimulate bone formation should be investigated. Development of novel therapies specific to alcoholics should also be encouraged.

Animal Models

Good models for chronic alcohol abuse have been established. However, improved animal models for binge and underage drinking should be developed and validated. While administration of high concentrations of alcohol to the diet of baby rats has clearly demonstrated that alcohol can antagonize bone growth, it is not at all certain that these findings are relevant to the important social and public health problems of underage drinkers. Specifically, there is an urgent need for models which better replicate the drinking patterns of adolescents, as well as moderate and binge drinking adults. Animals could be used to investigate the respective effects of total alcohol consumed, peak blood levels, age of initiation, and frequency and duration of consumption on bone and mineral metabolism. Such studies are likely to provide important insight into the variability observed in alcoholics and moderate drinkers and should be encouraged.

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FIGURE LEGEND

Figure 1: The bone remodeling cycle.

Time A: Quiescent phase - Inactive bone with surface lined with bone lining cells. Neither bone resorption nor formation is occurring on this region of bone surface.

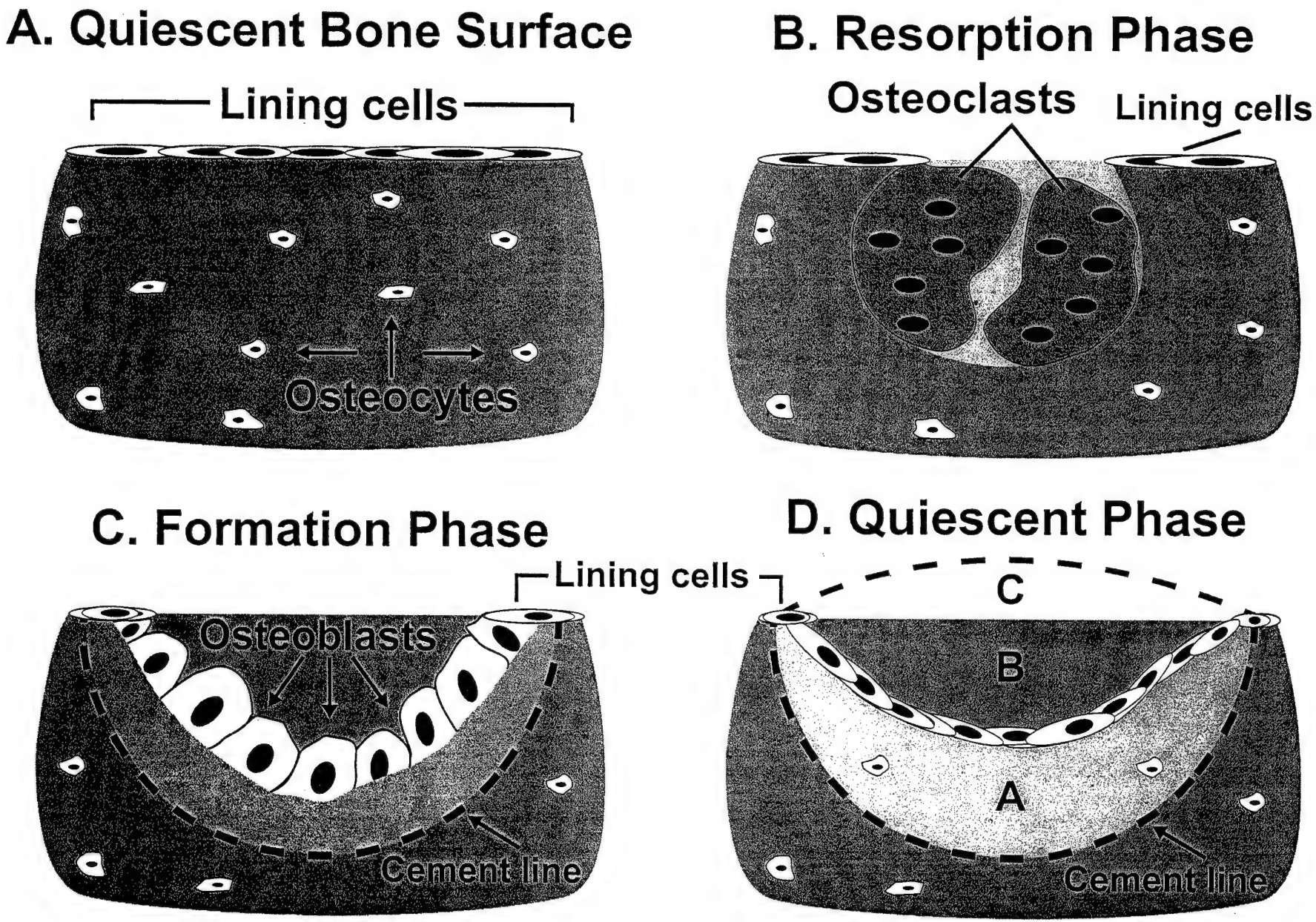
Time B: Resorption phase - Osteoclast mediated bone resorption. The osteoclasts remove a discreet packet of bone, creating a lacunae.

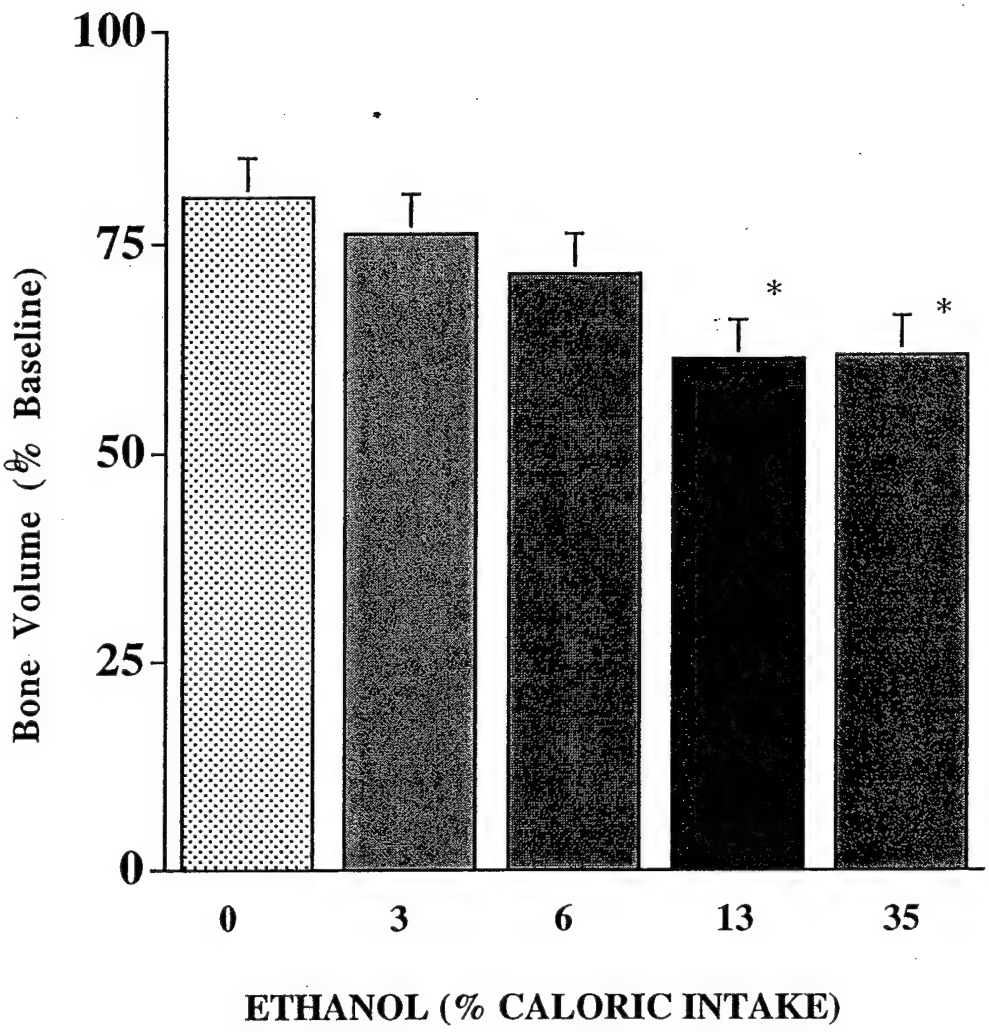
Time C: Formation phase - Osteoblasts form bone matrix which falls in the lacunae. The cement line defines the boundary between the newly formed bone and the surface of the lacunae excavated by the osteoclasts at the end of the resorption phase.

Time D: Quiescent phase - Inactive bone surface showing the completed remodeling cycle. The new surface may be underfilled (a), exactly filled (b), or overfilled (c) the resorption lacunae reflecting a local decrease, no change or increase in bone mass, respectively. The most likely mechanism for alcohol-induced bone loss in adults is underfilling of the resorption lacunae during bone remodeling.

Figure 2: Dose response effects of alcohol on cancellous bone volume in female 8-month-old adult rats. A baseline group of animals was sacrificed at the start of the study. The treatment groups were fed a liquid diet containing ethanol comprising 0-35% of their caloric intake for 4 months. Cancellous bone volume in he treated groups was expressed as % baseline control group and the data analyzed by ANOVA. There was dose dependent decrease in cancellous bone

volume at the proximal tibial metaphysis over and above the age-related bone loss. Data are mean ± SE (n = 8-11); *$p<0.05$ compared to age-matched rats fed the 0% ethanol control diet.




100
75
50
25
0
Bone Volume (% Baseline)
*
*
0
3
6
13
35
ETHANOL (% CALORIC INTAKE)

Appendix 8

ANIMAL MODELS FOR OSTEOPOROSIS

Running Title: Animal Models

Key Words: Rats, dogs, monkeys, mice,
bone metabolism, fractures, aging

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Introduction

Laboratory animals have played a major role in the unprecedented recent improvements in the management of osteoporosis. They have contributed to enhanced knowledge of the etiology of osteoporosis. Additionally, animals have been essential for preclinical evaluation of the efficacy and safety of interventions intended to prevent and/or reverse bone fragility. The purpose of this review is to evaluate the strengths and weaknesses of the most well-established animal models for osteoporosis as well as the methods used to measure bone quantity, quality and turnover in animals. For a more comprehensive discussion of animal models, see Kimmel (1) and Geddes (2).

Goals of Animal Models for Osteoporosis

The ideal laboratory animal model would replicate a human condition with an absolute degree of fidelity. Unfortunately, this goal has not been achieved for osteoporosis, in part because fracture risk has not been reproduced in animals, and it has proven difficult to ascertain the true degree of correspondence between the mechanisms which lead to the bone changes in the animal model and its human counterpart.

Bone mass, although relatively easy to measure, is an insufficient endpoint for judging the fidelity of an osteoporosis model because fracture is the clinical outcome of osteoporosis and it is very important to determine the cause of the bone fragility leading to fracture. Osteopenia can be localized or general, involve cortical and/or cancellous bone, and result from an inhibition of bone growth as well as a net increase in bone resorption. An animal model which develops relative osteopenia because of a disturbance in bone growth is not valid as a model for adult onset bone loss. Similarly, species differences at the cellular and biochemical levels may negatively influence the usefulness of an animal model.

An impressive number of factors that influence bone metabolism have been reported (Figure 1) and certainly many others remain to be discovered. Consideration of the astronomical number of possible combinations of these factors leads to the conclusion that a similar phenotype could result from more than one distinct factor or combination. How then can we ever know the degree of correspondence of the mechanism(s) which mediate osteopenia in humans and laboratory animals?

The extent to which the underlying signaling pathways which regulate bone mass are conserved between species can rarely be known with certainty. It is, however, possible to objectively evaluate the usefulness of an animal model by evaluating the extent to which similar events, such as hormonal deficiency or aging, lead to similar metabolic, cellular and architectural changes in humans and the animal model. This approach is usually straight forward when applied to a single change (e.g., hormonal deficiency) but becomes much more difficult when trying to model complex processes such as aging. The ultimate test of an animal model's utility is its ability to successfully predict an outcome in people. A final consideration when evaluating animal models is practicality. High cost and limited availability will prevent the widespread adoption of an otherwise promising model.

The Use of Laboratory Animals as Models for Osteoporosis

Osteoporosis is a condition which is characterized by skeletal fragility caused by reduced bone mass and architectural defects. Osteoporosis is distinguished from other osteopenias in that the bone material properties are normal in osteoporotic bone. Bone fragility can result from numerous causes: gonadal hormone insufficiency, mechanical disuse, anti-inflammatory and immunosuppressant drug therapy, alcohol abuse, tobacco use and aging are examples of individual risk factors for osteoporosis that have been modeled in animals. Multifactoral animal models have also been developed to investigate age related bone loss as well as the genetic predisposition toward bone fragility.

Rodents, dogs, monkeys, and apes are the principal animals used to model osteoporosis. Each species has strengths and weaknesses and no laboratory animal species is equally suited to model all of the risk factors which are associated with osteoporosis.

Rodents

The rat is the most frequently used laboratory animal for studying osteoporosis and its popularity as a model has increased in recent years (Figure 2). Rats are chosen because they are inexpensive to purchase and maintain, grow rapidly, have a relatively short lifespan, have a well characterized skeleton, are widely available, and have proven to be excellent models for several of the most common risk factors for osteoporosis (Table 1).

The small size of a rat is a mixed blessing. The benefits mentioned above must be weighed against the disadvantages; which include the small blood volume for multiple biochemical measurements and small total amount of bone available, minimal intra-cortical bone remodeling, and size related difficulty of performing surgical procedures.

As a consequence of nonlinear scaling, bone mass decreases with size more than total body mass and cancellous bone mass decreases more than cortical bone mass. When compared to humans, the rat skeleton has proportionally less bone and a smaller contribution of cancellous bone to the total bone mass. The species difference in size can be important when evaluating biomarkers, bone turnover and total bone mass changes. For example, large relative changes in cancellous bone volume in the rat may result in relatively small changes in biomarkers and total bone mass.

The Growing Rat Model

A low peak bone mass is considered to be an important risk factor for development of osteoporotic fractures later in life. The growing rat is a useful model for evaluating the effects of endocrine, nutritional and other environmental factors on peak bone mass. The young rapidly growing rat is appropriate for studies designed to investigate factors related to peak bone mass but is a poor model for the adult human skeleton because skeletal growth is mediated by cellular processes which are not active in adults.

A growth curve for male Sprague Dawley rats is shown in Figure 3. As in humans, female rats grow more slowly than males and obtain a smaller peak bone mass (3). The rapid growth phase of the growth curve (Figure 3, region A) occurs during the first 6 months of life. Not only are the rats growing rapidly during this interval but the growth rate changes continuously. The rate of weight gain reaches a peak shortly after puberty (6 weeks of age) and then declines rapidly with increasing age. The rapid growth phase is followed by much slower weight gain (Figure 3, region B).

The Skeletally Mature Rat Model

The authors have frequently witnessed the unsubstantiated criticism of the rat as a model for the adult human skeleton, at scientific meetings and in manuscript and grant reviews, because of alleged

continuous bone elongation and lack of bone remodeling in this species. Despite the frequency of these criticisms, we have been unable to identify any experimental studies reporting either life-long growth of rat bone or an inability of this species to remodel bone. Indeed, there is compelling evidence for basic multicellular unit-(BMU) based endocortical and cancellous bone remodeling in rats (4). There is also conventional x-ray imaging-based evidence for epiphyseal closure in this species; the precise timing of which was found to be bone- and growth plate-dependent (5). Using fluorochrome labeling, a more sensitive method, the authors have not been able to consistently detect longitudinal bone growth in hindlimb long bones of female rats over 8 months old or in male rats over 12 months old. Bone elongation therefore appears to be largely confined to the initial third of a rat's expected lifespan, a relative growth period which is longer than in humans, but not markedly so. High resolution micro-computer tomography (μCT) of aged female rats has verified the fluorochrome-based results by clearly demonstrating bone bridging between the metaphysis and epiphysis (6), rendering any small amount of residual growth plate cartilage incapable of mediating longitudinal bone growth. These observations support use of the rat as a model for the adult human skeleton provided that either animals with fused growth plates are used, or it is demonstrated experimentally that growth does not influence interpretation of the data.

Rat Models for Osteoporosis

The observation that acute ovarian hormone deficiency leads to elevated cancellous bone turnover dramatically increased interest in the rat as a model for post menopausal osteoporosis (7). Subsequent studies showing that ovariectomy results in cancellous and cortical bone loss have lead to the widescale adoption of this model (3). Similar bone loss can also be induced in female rats by LHRH agonists and estrogen receptor antagonists (8,9). These alternatives to ovariectomy are reversible and have proven to be very useful for investigation of the bone loss associated with endocrine treatment of endometriosis. The predictive value of the ovariectomized rat is illustrated by the initial recognition of tissue selective actions of tamoxifen and other estrogen receptor ligands in that model. Subsequent confirmation in

humans and development of selective estrogen receptor modulators (SERMs) for prevention of osteoporosis were a direct result of the initial animal observations (10,11).

The cancellous osteopenia which occurs following ovariectomy in rapidly growing rats is primarily due to altered bone growth and thus is mediated by a mechanism which differs significantly from post-menopausal bone loss (12). In contrast, ovariectomy of skeletally mature rats is similar to menopause in that the surgery leads to cancellous and endocortical bone loss which is due primarily to abnormal bone remodeling.

The rat has been extensively used as a model for disuse osteoporosis. Disuse has been induced by unilateral sciatic nerve section, tendonotomy, unilateral limb casting, hindlimb unloading and spaceflight (13-17). Each of these seemingly dissimilar methods results in similar skeletal changes, implying that the effects on bone are primarily due to unloading. These rat models have been used to study the etiology of disuse osteoporosis in growing and mature as well as to evaluate the efficacy of potential interventions.

Alcohol abuse is one of the most important "life style" risk factors for osteoporosis. The histological changes in the skeleton of alcohol-dependent rats (18) were later identified in alcoholics providing evidence that the species will be useful for predicting human outcome. The major use of this model has been to better understand the etiology and severity of alcohol-induced bone loss (19).

Generalized age-related bone loss begins in men and women during their fifth decade, continues unabated through the remainder of life, and ultimately is responsible for senile osteoporosis. There is no compelling evidence that similar bone loss occurs in rats. However, localized bone loss may occur in the rat and aged gonadectomized rats develop severe osteopenia (6).

The usefulness of the rat as a model for glucocorticoid-induced osteoporosis is unclear. It is well established that glucocorticoids inhibit overall growth and bone formation in the rat. As a result, young rats may develop osteopenia relative to normal growing controls. However, this relative osteopenia does not accurately model glucocorticoid-induced bone loss in adult humans. Some rat studies have shown an inhibition of bone remodeling by glucocorticoids, leading to a local increase in bone mass (20,21). The transient increase in bone resorption and rapid severe bone loss which characterizes the pathogenesis of

glucocorticoid-induced osteoporosis in humans are generally not apparent in rats. There have been associations between glucocorticoids, increased bone resorption, and reduced bone density (22,23), but definitive reports of bone loss are lacking. Interpretation of these results may have been complicated by the inhibitory effects of high doses of glucocorticoids on reproductive hormones (24). The many discrepancies in the published literature suggest that the effects of glucocorticoids on bone turnover in the rat are inadequately understood to confidently recommend this species as a model for steroid-induced osteoporosis. Unfortunately, no other animals are clearly better.

Mouse

The mouse has similar growth characteristics to the rat. At 1/10 the mass of the rat, the advantages and disadvantages of its small size are even more pronounced.

The mouse is the premier laboratory animal model for studying the genetic contribution to peak bone mass and age-related bone loss (25). There are numerous well characterized mouse strains with differences in bone mass and response to co-morbidity factors. Additionally, transgenic technology allows the purposeful manipulation of specific gene expression (26). There is a long and growing list of transgenic mice with perturbed bone metabolism (Table 2). Improvements in the ability to dynamically regulate genes in specific cell types will further increase the power of the mouse model. These genetic manipulations are not without pitfalls when applied to osteoporosis. Demonstration that a gene is associated with bone mass in the mouse does not necessarily mean that it has any role in the pathogenesis of osteoporosis. It will be essential to demonstrate that there is a cause and effect relationship in humans.

Ovariectomy results in cancellous osteopenia and accelerated bone turnover in the mouse. However, there are also clear differences between human and mouse physiology regarding the actions of estrogens and estrogen analogs such as tamoxifen (27). While these species differences do not necessarily contraindicate its use, the ovariectomized mouse model should be approached with extreme caution.

The mouse is a promising model for age, disuse, and possibly even glucocorticoid-induced osteoporosis (28-30). However, these models have been inadequately characterized to recommend without qualifications.

Dog

Rodents are generally not suitable as models for intracortical bone remodeling. Larger animals such as the dog are more appropriate for these studies because they have well developed Haversian remodeling. The large animal also has major advantages as a model for highly localized bone fragility such as that associated with stress shielding by orthopedic implants (31). The dog is also a well established laboratory animal model for more generalized disuse. In contrast, the dog is not widely used as a model for postmenopausal osteoporosis. Whereas some investigators have detected bone loss following ovariectomy, with or without concurrent hysterectomy, other investigators have detected no changes (1-3). The relative insensitivity and inconsistent response of the dog skeleton to decreased gonadal hormones may be due to the six month interval between periods of luteal activity. The large size and relatively long life span also discourage the use of the dog model because of the increased cost of maintaining the animals as well as administration of larger quantities of expensive and/or dangerous chemicals. An additional consideration is the reduced availability of molecular probes specific to dogs compared to rats and mice.

Primates

Several species of monkeys and apes have been used as models for osteoporosis. Monkeys and apes are generally more similar to human physiology than the more commonly used animal models for osteoporosis. The most compelling evidence for age-related osteopenia in an animal model is in monkeys (32). Unfortunately, bone loss was not observed in monkeys less than 30 years old, severely limiting the practical application of what otherwise would be an excellent model for aging. Monkeys are an established model for disuse but they do not offer many significant advantages over the dog (33). One advantage that monkeys and apes have over other large animals is the availability of molecular probes. Because of the species similarity, many human probes are suitable for use in monkeys. Ovariectomy

results in bone loss in monkeys raised in captivity (34). However, recent studies in monkeys reared primarily in the wild failed to demonstrate bone loss following ovariectomy. The use of monkeys and apes as a model for osteoporosis is greatly limited by their expense, long life span, limited availability, and ethical concerns.

Evaluation of the Osteopenic Skeleton of Animal Models

The choice of methods to evaluate bone mass, architecture and metabolism are as important as the choice of the animal model. Endpoints which are routinely studied in human subjects are useful for evaluating the fidelity of the animal model. The principal purpose of the animal model, however, is to extend knowledge beyond that which can be obtained in humans by employing more sophisticated and/or invasive methods than are generally available to human studies.

Densitometry – Single and dual photon densitometry are commonly used in laboratory animals (15,35) to measure bone mineral density (BMD) and bone mineral content (BMC). The resolution has improved steadily during the last decade to where it is now possible to measure individual bone compartments in animals as small as a mouse. BMD measurements reported in animals are generally assumed to be comparable to those routinely obtained in adult human subjects and changes are usually interpreted as reflecting changes in bone mass. This interpretation is often not valid. BMD has no fundamental physical meaning; BMD is the mineral content of the bone normalized to an apparent cross-sectional area. Interpretation of BMD changes in growing animals is especially difficult because cross-sectional area and mineral content are both changing over time and are often differentially influenced by treatment (e.g., ovariectomy). As a result, BMD and bone mass can change in opposite directions.

In contrast, densitometry provides quantitative measurement of material (BMC) which is directly related to bone mass. Because of the inherent difficulties of interpreting BMD, BMC is the more informative endpoint and should always be reported.

The recent application of peripheral computerized tomography (pQCT) and high-resolution micro-computer tomography (μCT) to assess bone changes in living animals provides the investigator

with powerful new imaging techniques (36). These instruments are capable of significantly higher resolution than currently used densitometric methods. The 3-dimensional architecture of bone can be studied over real time in small animals at a resolution capable of visualizing individual trabeculae. However, reconstructing images using voxal (3-dimensional equivalent of a pixel) dimensions which are similar in length to trabecular thickness cause errors, due to partial volume effects, greatly limiting the amount of architectural information that can be derived from these reconstructions. Radiation exposure, which increases as a cubic function compared to a linear increase in resolution, is the principal factor limiting further improvements in resolution in living animals.

Much higher resolution can be obtained when μCT is applied to tissues ex vivo because high radiation exposure is no longer a concern. Detailed 3-dimensional architectural measurements can be obtained from the reconstructions as well as bone density. Mechanically loaded bone regions as small as individual trabeculae can be compared to the unloaded bone and the mechanical properties can be calculated after measuring the deformation, providing an alternative method to finite element modeling. Recent studies indicate that μCT can detect local changes in bone density where bone seeking heavy metals have been deposited following acute administration. This finding suggests that in the future μCT can be used to measure bone formation in 3-dimensions using principles analogous to fluorochrome-based light microscope 2-dimensional measurements (36).

Biochemical Markers – Analysis of mineral homeostasis can be performed in laboratory animals more easily than in humans, using a variety of in vivo and ex vivo approaches. The mineral (Ca, P, Mg) content of blood and urine are easily measured and radioisotopes can be administered as tracers. Additionally, ex vivo studies can be used to extend the capabilities of human studies to evaluate transport of minerals across the intestinal mucosa.

In contrast, the availability of biochemical markers of bone metabolism is generally more limited for animal models than humans. As a consequence, human assays have frequently been adapted to animals with loss of specificity and sensitivity. Markers for osteoblast differentiation and activity (alkaline phosphatase and osteocalcin) and collagen breakdown products are the most common

biomarkers of bone metabolism. These markers are useful for indirect detection of changes in bone metabolism and mineral homeostasis at the level of the whole organism. Since the same measurements are routinely performed in humans, a direct comparison between the human and animal model can be made. Also, repetitive collection of blood and urine to establish a time course can be made in most laboratory animals.

There are several important limitations of biochemical markers. They provide no information regarding bone mass and strength. They do not distinguish between the appendicular and axial portions of the skeleton nor between cortical and cancellous bone. As a result, biomarkers may not detect important localized changes in bone metabolism. Finally, interpretation of biochemical markers must be made with great caution in rapidly growing animals as well as in severely osteopenic animals because changes in age and bone volume will influence levels of biomarkers. Because of the limitations, biochemical markers are best used as an adjuvant to methods which directly evaluate bone mass and regional bone turnover.

Histomorphometry – Histology can be used to provide a two-dimensional assessment of bone mass and architecture (7,10,14,16,17,38). The method has much greater resolution than densitometry and most alternative imaging techniques. One of the most powerful applications of histology is the use of fluorochrome labeling techniques to estimate changes in bone formation. This approach is called dynamic histomorphometry and is exquisitely sensitive because the fluorochromes act as time markers which can be used to limit the measurements to exclude bone that was formed prior to the treatment interval. Histology is the only routine method for estimating bone cell number. Osteoclast, preosteoblast, osteoblast, lining cell and osteocyte number can be measured directly in histological sections. Changes in osteoblast activity can be estimated from measurements of cell number and dynamic measurements. There is no well-established comparable dynamic index for bone resorption but changes in the rate of bone resorption and osteoclast activity can be inferred from the net change in osteoclast number, bone volume, and osteoblast activity. In some cases, it is possible to estimate changes in the rate of bone resorption by measuring retention of a fluorochrome label (38).

Histomorphometry has important limitations that are related to the small amount of tissue measured. Most histomorphometric measurements are normalized to a tissue sampling area. This approach is only valid when the sampling site is comparable in all of the groups. This requirement is difficult to accomplish when comparing animals of differing ages or growth rates. Histological changes at one sampling site should not be extrapolated to other skeletal sites. Densitometry, pQCT, μCT, and RNA analysis are methods that complement bone histomorphometry, for evaluating bone mass, architecture and cell activity, respectively (25,34-39).

Histomorphometry can be performed at essentially any skeletal site and theoretically information on global changes to the skeleton could be obtained using this method. However, tissue preparation and analysis is sufficiently time consuming that for practical reasons histomorphometry is limited to evaluation of a relatively small number of sites. Most investigations focus on hindlimb long bones or lumbar vertebrae, which are representative of the appendicular and axial skeleton, respectively.

Molecular Histomorphometry – Molecular histomorphometry couples the ability of conventional histology to resolve individual cells within tissue sections and molecular techniques to detect the presence of specific molecules within a cell. Initial studies in molecular histomorphometry involved the localization of radiolabeled amino acids and nucleotides by autoradiography. Immunohistochemistry and in situ hybridization provide important new tools for localizing gene products to specific cells.

Mechanical Testing – Bone strength is rarely measured in humans but is assumed to be an important risk factor for osteoporotic fractures. A relationship between bone strength and fracture risk has not been established in animal models because of low fracture rates. Nevertheless, measurement of bone mechanical properties is an important tool for evaluating the functional significance of changes in bone mass and/or architecture.

Three-point bending, four-point bending and torsion testing are the most common methods of measuring bone mechanical properties. These measurements are performed at the midshaft diaphysis, a site at which osteoporotic fractures are uncommon. Compression testing of vertebrae and cantilever

testing of the head of the femur have been developed. These newer techniques are highly recommended because they more closely approximate the type of failures associated with osteoporotic fractures (40).

Fracture Repair – Well-characterized animal models for fracture healing have been developed (41), but fracture repair studies are not routinely performed in osteoporotic animal models. Such studies are urgently needed because impaired fracture repair can dramatically increase morbidity in elderly patients. Animal models can be used to investigate the effects of age, hormones and life-style choices on fracture repair. Existing and future treatments may significantly reduce the risk of osteoporotic fractures but there is no immediate likelihood that intervention will completely prevent fractures. It is therefore imperative that potentially therapeutic interventions for osteoporosis be carefully investigated in animal models to also evaluate its effect on fracture healing.

Cell Biology – Animal models for osteoporosis can provide a source of cells for in vitro studies designed to evaluate potential changes in the composition and/or proliferative capacity of bone cell populations. Isolated cells can also be profitably used for studies of disturbed signaling pathways.

Summary

Animal models will continue to be important tools in the quest to understand the contribution of specific genes to establishment of peak bone mass and optimal bone architecture, as well as the genetic basis for a predisposition toward accelerated bone loss in the presence of co-morbidity factors such as estrogen deficiency. Existing animal models will continue to be useful for modeling changes in bone metabolism and architecture induced by well-defined local and systemic factors. However, there is a critical unfulfilled need to develop and validate better animal models to allow fruitful investigation of the interaction of the multitude of factors which precipitate senile osteoporosis.

Well characterized and validated animal models that can be recommended for investigation of the etiology, prevention and treatment of several forms of osteoporosis have been listed in Table 1. Also listed are models which are provisionally recommended. These latter models have potential but are inadequately characterized, deviate significantly from the human response, require careful choice of strain or age, or are not practical for most investigators to adopt. It cannot be stressed strongly enough that the

enormous potential of laboratory animals as models for osteoporosis can only be realized if great care is taken in the choice of an appropriate species, age, experimental design, and measurements. Poor choices will result in misinterpretation of results which ultimately can bring harm to patients who suffer from osteoporosis by delaying advancement of knowledge.

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Table 1: Animal Models for Investigation of the Etiology, Prevention and Treatment of Osteoporosis		
Model	**Recommended**	**Conditionally Recommended**
Establishment of peak bone mass	Mouse, rat	
Genetic contribution to osteoporosis	Mouse	
Sexual dimorphism of skeleton	rat	Mouse[1]
Post-menopausal osteoporosis	Rat	Dog[2], mouse[3], primate[4]
Fracture repair	Dog, rat	
Disuse	Dog, rat	Mouse[2]
Steroid-induced bone loss		Mouse[2], rat[2,3]
Alcohol abuse induced osteoporosis	Rat	
Senile-related bone loss		Mouse[1,2], rat[2,3], primate[4]

[1] strain dependent
[2] insufficient characterization and/or controversial
[3] important differences compared to human response
[4] impractical for most investigators

Figure Legends

Figure 1: Factors implicated in bone metabolism.

Figure 2: A compilation of publications using animals as models for osteoporosis between 1966 and 1998. The number of publications is shown by year for rats, mice, dogs and monkeys.

Figure 3: A representative curve for the male Sprague Dawley rat. Region A corresponds to the rapid growth interval. Region B corresponds to the slow growth interval. Rats gradually increase in weight following cessation of skeletal growth (not shown) which occurs in the male at about 12 months of age and somewhat earlier in the female.

Table 2. Some transgenic mouse models exhibiting perturbed bone metabolism

KNOCK-OUT	SKELETAL PHENOTYPE	OVER-EXPRESSION	SKELETAL PHENOTYPE
Activating Transcription Factor-2 (ATF-2)	Dwarfish, chondrodysplasia	BMP Receptor	Retarded ossification
Alkaline phosphatase	Decreased NOc; resembling infantile hypophosphatemia (rickets, osteomalacia)	Brain natriuretic peptide	Enhanced endochondral ossification, elongated growth plate
c-ab1	Reduced NOb and AOb; similar to Type II osteoporosis	Calcitonin gene-related peptide	Increased bone density
Bcl-2	Dwarfism; pseudo-woven bone formation	Cyclin D1	Chronic to mild to moderate hyperparathyroidism.
Biglycan	Reduced BMD, thinner cortex – osteopenia	FGF Receptor 3	Dwarfism
Bone sialoprotein	Reduced ossification, abnormally large incisors, smaller suture and bone marrow spaces in calvaria	c-fos-jun (double)	Higher frequency of osteosarcomas
Cathepsin K	Osteopetrosis	c-fos	Osteosarcomas, chondroblastic
Ca Sensing Receptor	Stunted skeletal growth, reduced cortex; multiple fractures-osteopenia	HTLV-1	Increased bone turnover resembling Paget's disease

KNOCK-OUT	SKELETAL PHENOTYPE
Cbfa1/PeBp2 αA	-/- Fatal; open fontanelles & sutures; blocked intramembranous and endochrondral ossification
CSF 1 (macrophage)	Osteopetrosis
Dopamine transport gene	Smaller cross-sections and lengths of long bones, diminished mechanical strength
Estrogen Receptor alpha	Bone loss with OVX; no phenotype in SHAM mouse; reduced femur lengths in males
Estrogen Receptor beta	Increased cortical cross-sectional area and BMC of femur; unchanged cancellous bone density
c-fos	Osteopetrosis
FGF Receptor 3	Kyphosis & scoliosis; accelerated & prolonged bone growth

OVER-EXPRESSION	SKELETAL PHENOTYPE
Human Growth Hormone	Increased BMD, cortex and mechanical strength
ß1 Integrin	Excessive bone resorption & increased NOc on endocranial surfaces
IL4	Cortical & cancellous osteopenia
IL-6	Hypercellularity, focal cell proliferation, distorted bone formation in calvaria
Measles Virus Receptor	Increased NOc
PTHrp-Collagen II	Jansen's metaphyseal chondrodysplasia, delayed endochondral ossification
TGFß1	Decreased BMC, reduced bone connectivity

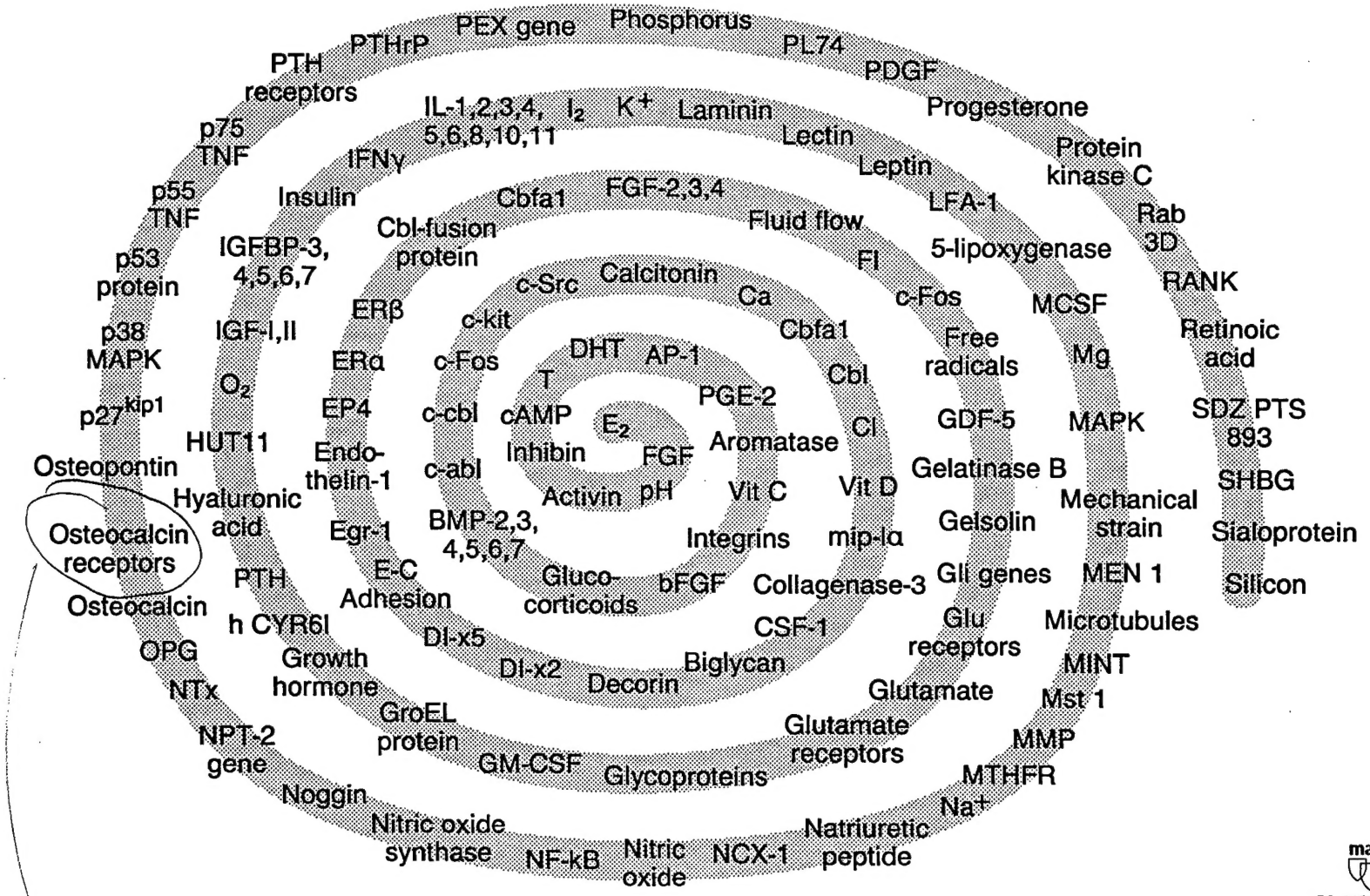
KNOCK-OUT	SKELETAL PHENOTYPE
24-hydroxylase	In 2nd generation homozygotes osteomalacia at sites of intramembranous ossification
Interleukin 1 Receptor	Protection against OVX-induced bone loss
Link Protein (Crtl 1)	Dwarfism; craniofacial abnormalities
Matrix Gla Protein	Extensive calcification
Matrix metalloproteinase 14	Dwarfism; osteopenia
5-lipoxygenase	Thicker cortex; partial protection against OVX-induced bone loss; stiffer but more brittle bones
Npt2, renal specific	Resembling Hypophosphatemic rickets
NFκß	Osteopetrosis
oim (-/+)	Reduced mechanical strength-mild (type 1) osteogenic imperfecta.
Osteocalcin	Defective bone maturation

OVER-EXPRESSION	SKELETAL PHENOTYPE
TGFß2	Increased bone turnover leading to bone loss
TRAF6	Osteopetrosis
Truncated TGFß2 Receptor	Increased cortex, cross-sectional area, trabeculae
Thymidine Kinase	Osteoblast ablation-osteopenia
TNF Receptor Fusion Protein	Protection from OVX-induced bone loss
Vitamin D Receptor	Increased cross-sectional area, mechanical strength

KNOCK-OUT	SKELETAL PHENOTYPE	OVER-EXPRESSION	SKELETAL PHENOTYPE
Osteoprotegerin	Osteoporosis		
RANKL	Osteopetrosis		
Procollagen Type II alpha 1 (Col2α1)	Absence of endochondral ossification		
Prolactin Receptor	Reduced mineral apposition rate		
PTH-PTHrp and PTH-PTHrp Receptors	Osteochondrodysplasia		
Pu.1	Osteopetrosis		
Src	Osteopetrosis		
Tartrate-resistant Acid Phosphatase	Osteopetrosis		
Thrombospondin 2	Increased cortex		
TNF Receptor-associated Factor 6 (TRAF6)	Osteopetrosis		
TGFß	Reduced BMD, trabecular connectivity.		
Vitamin D Receptor	Impaired bone formation; similarities to Type II Rickets		

Abbreviations:
AOb - osteoblast activity
BMC – Bone Mineral Content
BMD – Bone Mineral Density
BMP - Bone Morphogenetic Protein
Cbfa1 – Core binding factor A1
CSF 1 – Colony-stimulating Factor 1
FGF – Fibroblast Growth Factor
fos – transcription factor
HTLV - Human T cell leukemia virus
IL4 and IL6 – Interleukin
jun – transcription factor
Npt2 - Renal-specific sodium phosphate cotransporter
NFκß - transcription factor, nuclear factor kappa-B
NOc - Osteoclast number
NOb - Osteoblast number
OVX - ovariectomy
ORX - orchitectomy
oim –single base deletion in proα2(I) collagen gene
PTH-PTHrp - Parathyroid Hormone -Parathyroid Hormone Related Protein
RANKL - Osteoprotegerin Ligand
SHAM – sham-operqted
Pu.1 - hematopoietic transcription factor
TGFß - Transforming Growth Factor ß
TNF - Tumor necrosis factor
TRAF6 – TNF receptor-associated Factor 6

Some of the Players in the Game



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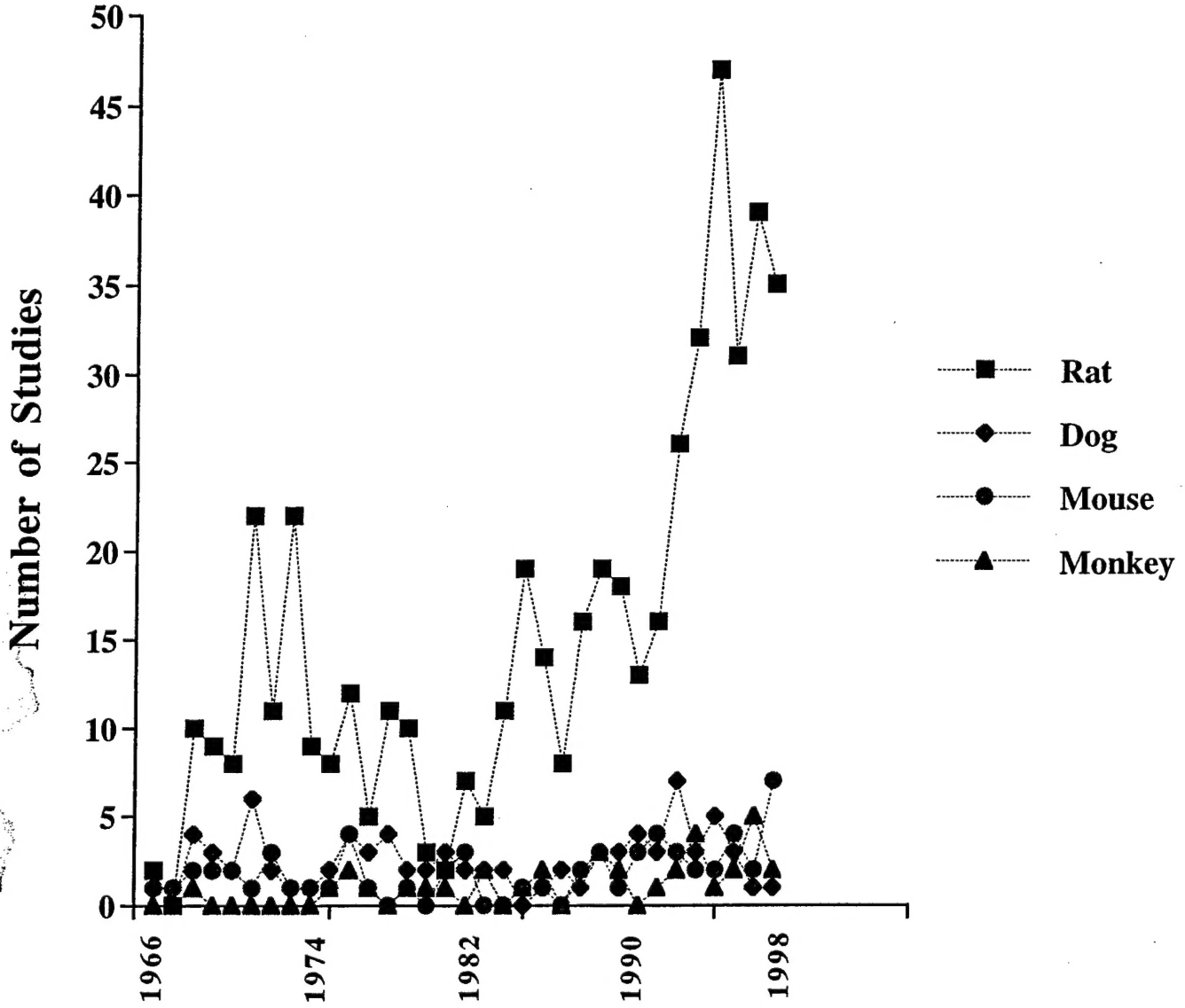
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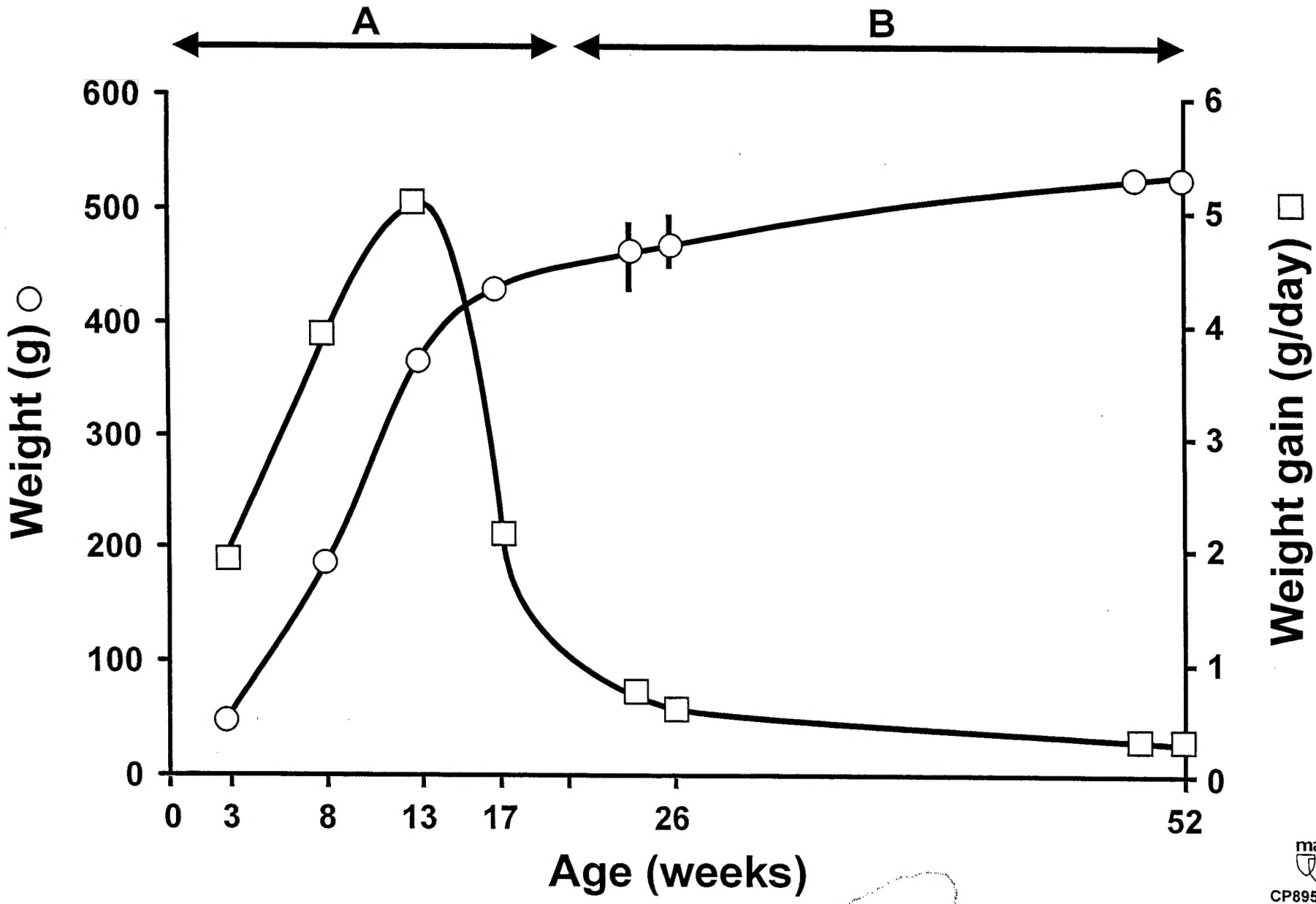

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Appendix 9

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Chronic alcohol abuse is a major risk factor for osteoporosis but the effects of moderate drinking on bone metabolism are largely uninvestigated. Here we studied the long-term dose response (0,3,6,12, and 35% caloric intake) effects of ethanol on cancellous bone in the proximal tibia of eight-month-old female rats. After four months of treatment, all doses of ethanol had decreased bone turnover. The inhibitory effects of ethanol on bone formation were dose dependent. A reduction in osteoclast number occurred at the lowest dose rate but there were no further reductions with higher concentrations of ethanol. An imbalance between bone formation and bone resorption at higher dose rates of ethanol resulted in trabecular thinning. Ethanol had no significant effect on osteoblast perimeter, and except at the highest dose rate did not reduce mRNA levels for bone matrix proteins. These data suggest that the suppression of bone formation by moderate alcohol consumption was largely due to a post-transcriptional disturbance in regulation of bone matrix protein synthesis and/or deposition. mRNA levels for IL-6 and IGF-I were reduced suggesting that these cytokines may play a role in mediating the decrease in bone turnover in alcohol consuming rats. Our observations in rats raise the concern that moderate consumption of alcoholic beverages in humans may reduce bone turnover and potentially have detrimental effects on bone mass, architecture and strength.

Cluster: 63 Osteoporosis Pathophysiology: Miscellaneous

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